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Creatine feeding increases GLUT4 expression in rat skeletal muscle

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Ju, Jeong-Sun, Jill L. Smith, Peter J. Oppelt, and Jonathan S. Fisher. Creatine feeding increases GLUT4 expression in rat skeletal muscle. *Am J Physiol Endocrinol Metab* 288: E347–E352, 2005. First published October 19, 2004; doi:10.1152/ajpendo.00238.2004.—The purpose of this study was to investigate the potential role of creatine in GLUT4 gene expression in rat skeletal muscle. Female Wistar rats were fed normal rat chow (controls) or chow containing 2% creatine monohydrate ad libitum for 3 wk. GLUT4 protein levels of creatine-fed rats were significantly increased in extensor digitorum longus (EDL), triceps, and epitrochlearis muscles compared with muscles from controls ($P < 0.05$), and triceps GLUT4 mRNA levels were ~100% greater in triceps muscles from creatine-fed rats than in muscles from controls ($P < 0.05$). In epitrochlearis muscles from creatine-fed animals, glycogen content was ~40% greater ($P < 0.05$), and insulin-stimulated glucose transport rates were higher ($P < 0.05$) than in epitrochlearis muscles from controls. Despite no changes in [ATP], [creatine], [phosphocreatine], or [AMP], creatine feeding increased AMP-activated protein kinase (AMPK) phosphorylation by 50% in rat EDL muscle ($P < 0.05$). Creatinine content of EDL muscle was almost twofold higher for creatine-fed animals than for controls ($P < 0.05$). Creatine feeding increased protein levels of myocyte enhancer factor 2 (MEF2) isoforms MEF2A (~70%, $P < 0.05$), MEF2C (~60%, $P < 0.05$), and MEF2D (~90%, $P < 0.05$), which are transcription factors that regulate GLUT4 expression, in creatine-fed rat EDL muscle nuclear extracts. Electrophoretic mobility shift assay showed that DNA binding activity of MEF2 was increased by ~40% ($P < 0.05$) in creatine-fed rat EDL compared with controls. Our data suggest that creatine feeding enhances the nuclear content and DNA binding activity of MEF2 isoforms, which is concomitant with an increase in GLUT4 gene expression.

acetyl-coenzyme A carboxylase; adenosine monophosphate-activated protein kinase; myocyte enhancer factor 2; phosphocreatine; creatinine

A NUMBER OF STUDIES regarding beneficial effects of creatine supplementation on muscle glucose metabolism have been reported. For example, creatine supplementation improves impaired glucose tolerance (9) and increases glycogen content (18). Furthermore, combined creatine and carbohydrate supplements result in a greater postexercise muscle glycogen resynthesis than carbohydrate alone (23). However, few studies (17, 18, 28) have been done regarding the effects of creatine supplementation on changes in the expression level of the insulin- and contraction-regulated glucose transporter (GLUT4) that mediates glucose uptake in muscle tissue, and a clear picture has not emerged from the findings described in the literature. It has been demonstrated that creatine supplementation prevents a decrease in muscle GLUT4 protein content during 2 wk of immobilization and increases GLUT4 protein content during a subsequent 10 wk of rehabilitation training in healthy subjects (18). However, the physiological mechanisms supporting elevated GLUT4 expression by creatine supplementation were not addressed. A separate study of creatine supplementation in humans found ~30% greater (not statistically significant) GLUT4 protein in muscle from creatine-supplemented subjects than from control subjects (28). There is reportedly no effect of 5 days of creatine supplementation on GLUT4 expression in rats (17).

A recent study demonstrated an approximately twofold increase in AMP-activated protein kinase (AMPK) phosphorylation after 48 h of creatine supplementation in L6 myocytes (5). It has been shown that myocyte enhancer factors 2 (MEF2)A, -C and -D, transcription factors that regulate GLUT4 expression in muscle (27), increase in response to AMPK activation (16). We hypothesized that the beneficial effects on glucose metabolism related to creatine supplementation might occur concomitantly with increased GLUT4 expression. We have evaluated whether 3 wk of oral creatine supplementation enhances GLUT4 biogenesis concomitantly with increased AMPK phosphorylation and MEF2 DNA binding activity.

MATERIALS AND METHODS

Materials. A polyclonal antibody specific for the GLUT4 glucose transporter was the generous gift of Dr. Mike Mueckler (Washington University, St. Louis, MO). The primers and the internal standard mRNA for competitive RT-PCR were kindly supplied by Dr. John Holloszy (Washington University). Horseradish peroxidase-conjugated goat anti-rabbit IgG was purchased from Pierce Biotechnology (Rockford, IL). MEF2A and -D antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). MEF2C, phospho-AMPKα (P-AMPK), AMPKα, and phospho-acetyl-CoA carboxylase (P-ACC) antibodies were purchased from Cell Signaling Technology (Beverly, MA). The antibodies against AMPK and P-AMPK are pan-α antibodies that bind to both isoforms, α1 and α2, of the catalytic subunit of AMPK. ATPlite and reagents for enhanced chemiluminescence (ECL) were obtained from Perkin-Elmer Life Sciences (Boston, MA). NE-PER Nuclear Extraction Reagent, Biotin 3′-End DNA Labeling Kit and LightShift Chemiluminescent EMSA (electrophoretic mobility shift assay) kit were purchased from Pierce Biotechnology (Rockford, IL). Creatinine amidohydrolase was obtained from MP Biomedicals (Philadelphia, PA). Radiolabeled 2-deoxyglucose and mannitol were obtained from American Radiolabeled Chemicals (St. Louis, MO). All other reagents were obtained from Sigma Chemical (St. Louis, MO).

Animal care. Fouteen female Wistar rats weighing ~90 g were obtained from Charles River Laboratories and divided into two groups: *1*) creatine (Cr) supplementation [2% creatine monohydrate

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Table 1. *Muscle high-energy phosphate metabolite and creatine content*

	Control	Creatine Fed
Creatine, μmol/g	6.1±0.4	6.8±0.7
PCr, μmol/g	9.5±0.7	10.0±0.8
TCr, μmol/g	15.4±0.7	16.4±1.1
[PCr]/[TCr]	0.61±0.002	0.61±0.023
Creatinine, μmol/g	1.6±0.2	2.9±0.3*
ATP, μmol/g	5.6±0.5	5.4±0.8
AMP, nmol/g	15.4±1.3	14.8±2.5

Values are means ± SE; n = 7/group. PCr, phosphocreatine; TCr, total creatine; [PCr]/[TCr], PCr-to-TCr concentration ratio. Extensor digitorum longus (EDL) muscles from either control rats or rats fed creatine were analyzed. *$P < 0.005$, control vs. creatine fed.

(Harlan Teklad, Madison, WI), n = 7 rats] and normal chow diet (n = 7 rats). The rats were provided with unrestricted access to water and diets for 3 wk. After an overnight fast, animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt), and the skeletal muscle tissues were rapidly dissected out. Epitrochlearis muscles were assayed for glucose transport activity, as described in *Glucose transport and glycogen assays*. Other muscles were immediately frozen with clamps cooled in liquid nitrogen. This research was approved by the Saint Louis University Animal Care Committee.

Metabolite analysis. Muscle samples were stored at −80°C until analyzed. For assay of ATP, phosphocreatine (PCr), creatine, and AMP, extensor digitorum longus (EDL) muscles were ground in 5.6% perchloric acid containing 40% ethanol at −17°C. The −17°C homogenization temperature was maintained by cooling grinding tubes in an ethanol-dry ice bath during homogenizations. Samples were spun and supernatants neutralized with buffer containing 3 M KOH, 0.5 M imidazole, and 0.4 M KCl. ATP was analyzed with the ATPlite Luminescence ATP Detection Assay System (Perkin-Elmer) according to the manufacturer's instructions. PCr, creatine, and AMP content were measured with spectrophotometric or fluorometric assays as previously described by Bergmeyer (2) and Passonneau and Lowry (20). Total creatine (TCr) content was obtained by adding PCr and creatine concentrations. Creatinine was assayed by an end point creatinine amidohydrolase method based on the kinetic assay described by Moss et al. (15).

Western blot analysis. Homogenates for Western blot analysis were made by grinding muscle samples with ice-cold buffer with grinding tubes resting in ice-water baths. Muscle extracts for GLUT4 analyses were prepared by homogenizing muscle in 250 mM sucrose containing 20 mM HEPES and 1 mM EDTA, pH 7.4 (22). For P-AMPK, AMPK, and P-ACC Western blots, muscle tissues were homogenized in a buffer suitable for preserving phosphorylation states of enzymes, containing 50 mM HEPES, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM Na_3PO_4, 100 mM NaF, 2 mM Na_3VO_4, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride (24), and samples for Western blots were prepared from supernatants after centrifugation of homogenates for 10 min at 14,000 *g*. For evaluation of MEF2 isoform protein content, nuclear extract from EDL muscle was isolated with

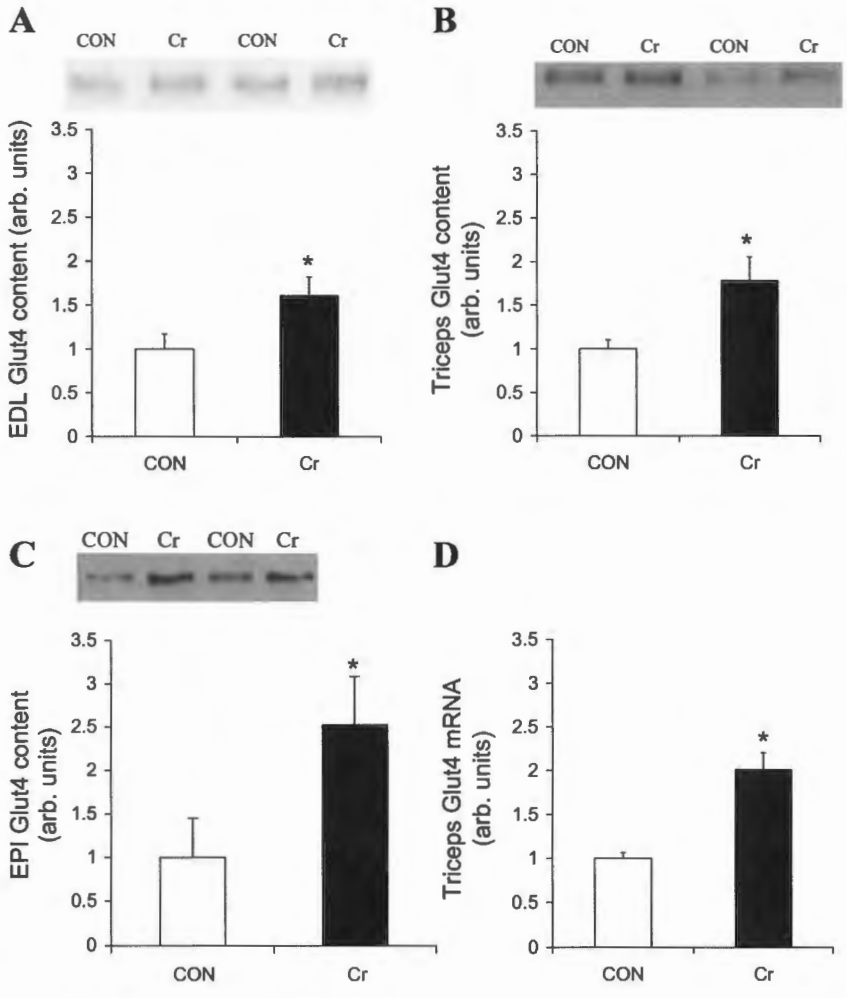

Fig. 1. Creatine feeding increases skeletal muscle GLUT4 expression. Muscle GLUT4 protein content was determined by Western blot analysis as described in MATERIALS AND METHODS for extensor digitorum longus (EDL; *A*), triceps (*B*), and epitrochlearis (EPI; *C*). *D*: intensities (arbitrary units) of triceps GLUT4 mRNA transcript from chow-fed control (CON) and creatine-supplemented (Cr) muscle samples. GLUT4 mRNA was measured in triceps muscles by competitive RT-PCR as described in MATERIALS AND METHODS. Values are means ± SE; n = 7/group for CON and Cr rats. *$P < 0.05$.

the NE-PER nuclear extraction reagent (Pierce). Protein concentrations in the samples were measured using a bicinchoninc acid protein assay reagent kit (Pierce). Aliquots of homogenates were solubilized in Laemmli sample buffer containing dithiothreitol and boiled for 5 min, except for samples for GLUT4 analysis, which did not contain dithiothreitol and were not boiled. Samples in Laemmli sample buffer were subjected to SDS-PAGE (10% resolving gels, 5% gels for P-ACC), Western blotting, and visualization with ECL. GLUT4, AMPK, P-AMPK, and P-ACC blots yielded single bands at the expected molecular masses. ACCβ (280 kDa) is the primary ACC isoform in muscle, but it is possible that if the α-isoform (257 kDa) was present, it could have run to the same position as the ACCβ band on P-ACC blots. The prominent band of the two shown on MEF2 blots is at the expected molecular mass.

Competitive RT-PCR. Triceps muscle was used for the GLUT4 mRNA analysis instead of EDL, because the mRNA extraction procedure required a larger amount of tissue than was available from EDL muscles. However, we believe that triceps is a good proxy for EDL for the mRNA analysis, because the fiber type distributions for the two muscles are similar (7). Total RNA from triceps muscles was isolated using TRIzol reagent (Invitrogen). Competitive RT-PCR was performed as described by Garcia-Roves et al. (12).

Glucose transport and glycogen assays. 2-Deoxyglucose (2-DG) glucose transport in the absence or presence of 60 μU/ml insulin was assayed as described by Young et al. (31). Epitrochlearis muscles were allowed to recover for 1 h after dissection at 35°C with gentle shaking in an incubation medium that consisted of 0.1% RIA-grade bovine serum albumin, 8 mM glucose, and 32 mM mannitol in Krebs-Henseleit bicarbonate buffer gassed with 5% CO_2-95% O_2. Muscles were then incubated for 30 min in the same medium in the absence or presence of 60 μU/ml insulin (Iletin II; Lilly, Indianapolis, IN) before being washed twice at 30°C for 10 min per wash in glucose-free medium containing 40 mM mannitol and insulin, if it had been present for the previous step. Finally, muscles were incubated at 30°C for 20 min in glucose-free medium containing 8 mM 2-DG (with 3 μCi/ml [^{3}H]2-DG) and 32 mM mannitol (with 0.2 μCi/ml [^{14}C]mannitol), with insulin present if it had been present in the previous incubations. Muscles were blotted and trimmed at 4°C and frozen with clamps cooled in liquid nitrogen. Muscle homogenates were made in 0.3 M perchloric acid, and intracellular 2-DG content was determined after scintillation counting, as described previously (31). Glycogen content for epitrochlearis muscles that were not exposed to insulin was assayed with the amyloglucosidase method described by Passoneau and Lauderdale (19).

EMSA. The MEF2 DNA binding activity assay was performed with nuclear extract from EDL muscle tissues prepared with the NE-PER nuclear extraction reagent (Pierce). Synthetic oligonucleotides were labeled with the biotin 3′-End DNA Labeling Kit (Pierce). This oligonucleotide probe contains a functional MEF2 binding site in the GLUT4 promoter (the recognition sequence for MEF2 is italicized): forward oligo, 5′-GAT CGC T*CT AAA AAT AAC* CCT GTC G-3′; reverse oligo, 5′-C GAC AGG *GTT ATT TTT AGA* GCG ATC-3′ (14). Reactions of biotin end-labeled target DNA and nuclear extracts in the presence or absence or 200-fold excess unlabeled oligonucleotides and subsequent electrophoresis and ECL visualization were performed with an EMSA kit (LightShift, Pierce Biotechnology) according to the manufacturer's instructions.

Data analysis. Data are presented as means ± SE. Creatine effects (creatine vs. control) were evaluated by a univariate analysis of variance (ANOVA). Glucose transport data were analyzed with a 2 × 2 ANOVA (insulin absence/presence × control/creatine).

RESULTS

Body weights and food intakes were measured every 2–3 days and did not differ between groups.

Muscle metabolites. EDL muscle high-energy phosphate metabolite contents were not altered by creatine feeding (Table 1). There were no significant differences in PCr, creatine, ATP, or AMP levels in rat muscle tissue between creatine-fed and chow-fed control animals (Table 1). The PCr-to-TCr concentration ([PCr]/[TCr]) ratio did not differ in EDL muscles from creatine-fed animals compared with muscles from controls (Table 1). However, creatinine levels were almost twofold greater in muscles from creatine-fed rats than in muscles from controls (Table 1). The creatinine concentrations for these muscles are in the ranges previously reported for rat skeletal muscle [~1 μmol/g, assayed after homogenization in water and mixing with trichloracetic acid (26)] and rat heart [~2.6 μmol/g, assayed after homogenization in perchloric acid (11)].

Muscle GLUT4 expression, glucose transport activity, and glycogen content. Creatine feeding induced an ~60% increase in GLUT4 protein in EDL muscle (Fig. 1*A*), an ~80% increase in GLUT content of triceps muscle (Fig. 1*B*), and a twofold increase of GLUT4 in epitrochlearis (Fig. 1*C*). As shown in Fig. 1*D*, triceps muscle GLUT4 mRNA levels increased by 100% in the creatine-fed group compared with chow-fed control animals, suggesting that increases in GLUT4 expression

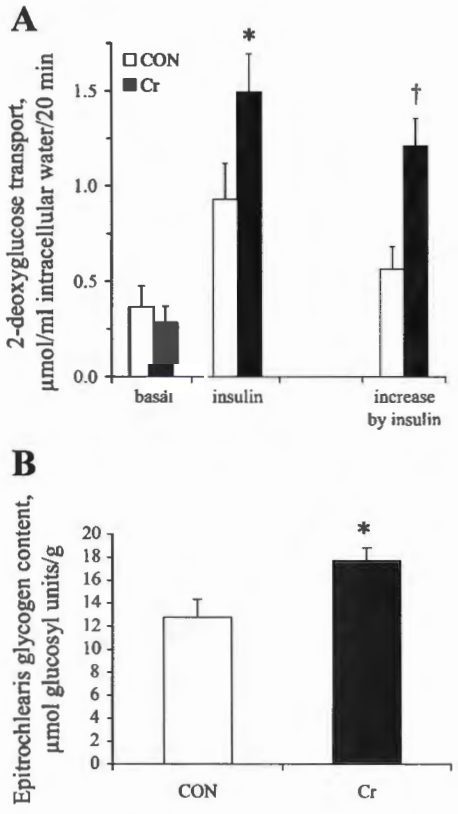

Fig. 2. Epitrochlearis muscle glucose transport activity and glycogen content. *A*: isolated epitrochlearis muscles from CON (n = 7 rats) and Cr animals (n = 6 rats) were assayed for glucose transport activity in the absence or presence of 60 μU/ml insulin and the insulin-stimulated increase in glucose transport, calculated as the difference between basal glucose transport and insulin-stimulated glucose transport for each animal. *B*: glycogen content of epitrochlearis muscles that were not exposed to insulin (n = 7/group). Values are means ± SE. *$P < 0.05$ for the creatine × insulin interaction in *A* greater insulin-stimulated glucose transport in creatine-fed animals than controls in *A*, and higher muscle glycogen content for creatine-fed animals than for controls in *B*. †$P < 0.005$ for greater insulin-stimulated increase in glucose transport for Cr animals than for CON.

induced by creatine supplementation are mediated at the transcriptional level. Because of the similar creatine-related increase in GLUT4 in EDL and triceps and the fiber type similarity between the two (7), we believe that the mRNA data for triceps are generalizable to EDL. Insulin (60 μU/ml) stimulated an ~2.5-fold increase in glucose transport rate in epitrochlearis muscles for control animals and an ~5-fold increase in glucose transport for creatine-fed animals (Fig. 2*A*). The creatine × insulin interaction (chow/creatine × basal/insulin) was statistically significant ($P < 0.05$), and insulin-stimulated glucose transport was greater for creatine-fed animals than for controls ($P < 0.05$). The data in Fig. 2*A* represent values for paired muscles (for each animal, glucose transport was assayed in both the absence and the presence of insulin). These muscle pairs allow computation of the increase in glucose transport that is stimulated by insulin for each animal, and this increase is about twofold higher for creatine-fed animals than for controls ($P < 0.005$). Glycogen content of epitrochlearis muscles was 40% higher for creatine-fed animals than for controls (Fig. 2*B*).

AMPK and ACC phosphorylation. The phosphorylation of Thr^{172} of AMPKα is shown in Fig. 3, *A* and *B*. AMPK phosphorylation of creatine-fed rats was significantly increased by 50% ($P < 0.05$) in EDL muscle compared with muscles from regular chow-fed rats. Muscle content of nonphosphorylated AMPK was not affected by creatine feeding (control 1.00 ± 0.02, creatine-fed rats 0.94 ± 0.08 arbitrary units, n = 7/group) Phosphorylation levels of ACC were 46% higher in EDL muscles from creatine-fed animals compared with EDL muscles from controls (Fig. 3, *C* and *D*). The increase in phosphorylation (Ser^{79}) of ACC, an AMPK substrate, is consistent with increased AMPK phosphorylation.

MEF2 isoform content. Nuclear content of MEF2A and MEF2D, members of the MEF2 transcription factor family known to regulate GLUT4 expression, was increased in the muscle of the creatine-fed vs. the control group (Fig. 4). Seventy-two percent higher levels of MEF2A (Fig. 4*A*) and 90% higher levels of MEF2D (Fig. 4*C*) in creatine-fed compared with control muscle were found. Concomitantly, MEF2C, another MEF2 isoform known to participate in the activity of GLUT4 promoter, was also increased by 60% in muscle nuclear extracts of the creatine-fed vs. the control group (Fig. 4*B*).

MEF2-DNA binding activity. MEF2 EMSA was performed using a synthetic oligonucleotide probe containing a sequence encoding for the GLUT4 promoter known to have a functional binding site for MEF2 isoforms. Three weeks of creatine feeding caused a 44% increase in MEF2 binding activity in nuclear isolates of EDL muscle compared with the control group (Fig. 5).

DISCUSSION

The present study demonstrated that 3 wk of creatine supplementation increases the expression of the GLUT4 gene in rat skeletal muscle. Creatine supplementation was related to increased phosphorylation of AMPK, increased content of MEF2 protein in nuclear extracts, and increased DNA binding activity of MEF2 isoforms.

The present study showed that creatine feeding increases AMPK phosphorylation in rat skeletal muscle. A recent study also shows that 48-h treatment with creatine increased activating phosphorylation of both AMPKα1 and -α2 isoforms (5). Currently, the effects of creatine supplementation on AMPK activation have not been fully studied. There is considerable evidence that AMPK activation is regulated by phosphorylation of the AMPK α-subunit by upstream kinases (AMP kinase kinase) (13, 29, 30) and that decreasing [PCr]/[TCr] ratio causes allosteric activation of AMPK (21). In our hands, creatine and phosphocreatine concentrations were not altered by creatine feeding. Although this is not a common finding (see, e.g., Ref. 17), other investigators have found no changes

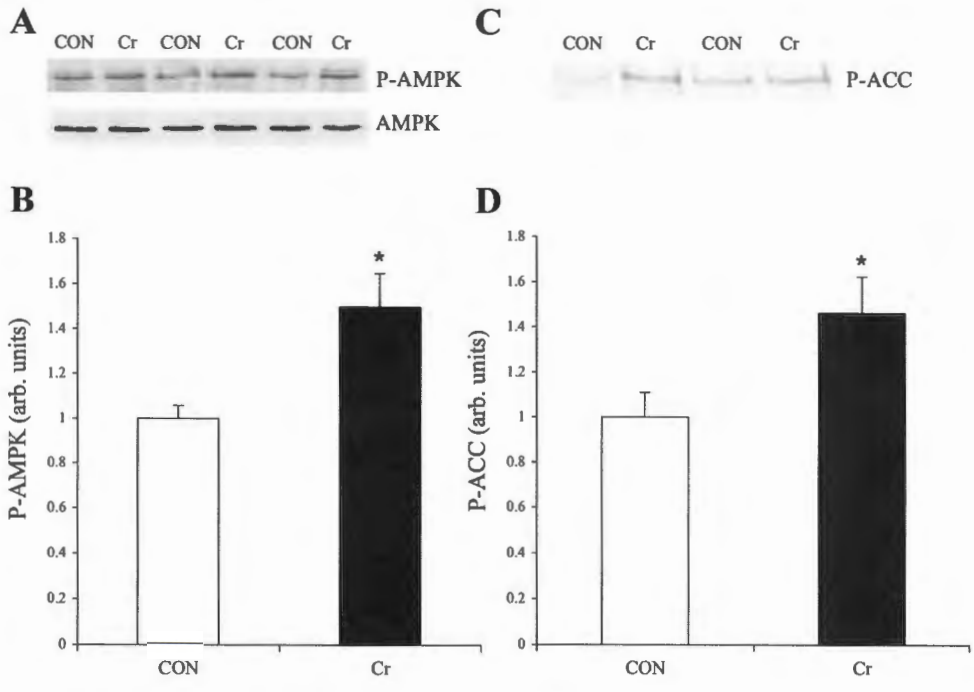

Fig. 3. Effects of creatine feeding on phosphorylation of AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) in EDL muscles. *A*: representative phospho-AMPK (P-AMPK, *top*) and AMPK (*bottom*) Western blot samples. *B*: densitometry of P-AMPK; n = 7/group for CON and Cr rats. C: representative sample of phospho-ACC (P-ACC) Western blots. *D*: densitometry of P-ACC; n = 7/group. *$P < 0.05$ vs. CON. Values are means ± SE.

in total creatine in skeletal muscle over 7 wk of creatine feeding (4). Interestingly, despite no changes in intracellular [creatine], increases in plasma [creatine], and no change in creatine transporter abundance, creatine uptake rates were lowered by ~50% in white muscle after the 7 wk of creatine feeding (4). This suggests that, during creatine feeding, some factor other than tissue total creatine content can affect muscle physiology. The present data demonstrating increased muscle creatinine concentrations after creatine feeding raise the question of whether muscle function can be altered by changes in the creatine plus creatinine pool, rather than creatine concentrations per se.

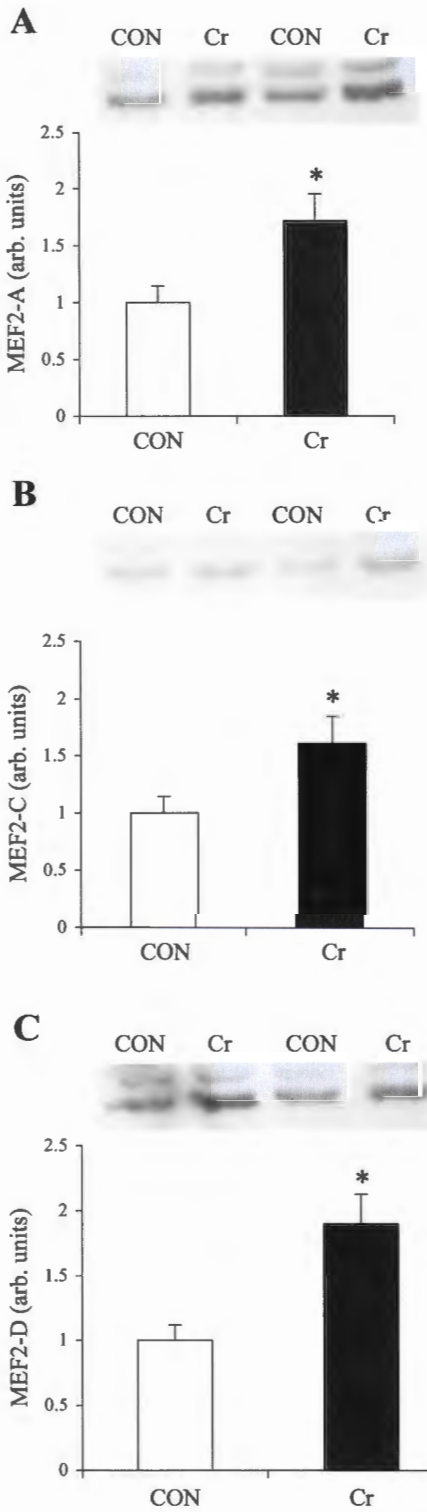

Fig. 4. Representative blots and their respective densitometric quantification of effects of creatine on myocyte enhancer factor 2 (MEF2) isoforms. Creatine feeding increased protein levels of MEF2 isoforms in Cr rat EDL muscle nuclear extracts. *A*: MEF2-A. *B*: MEF2-C. *C*: MEF2-D. Values are means ± SE; for 7/group for CON and Cr rats. $*P < 0.05$.

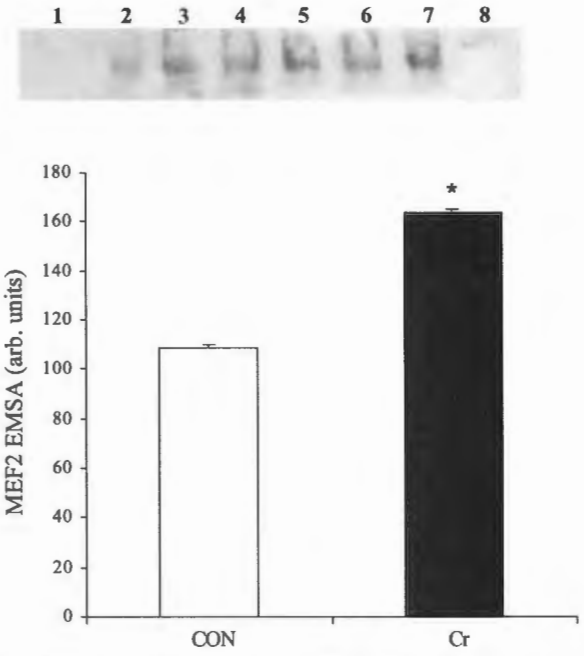

Fig. 5. MEF2 electrophoretic mobility shift assay from EDL muscle samples of rats fed either Cr or CON diet: DNA probe alone (*lane 1*), CON (*lanes 2, 4,* and *6*) and vs. Cr group (*lanes 3, 5,* and *7*), and an excess of unlabeled probe (*lane 8*). Values are means ± SE; $n = 7$/group. $*P < 0.05$.

In disagreement with the present study and the work of others (4) demonstrating that creatine feeding does not alter creatine content in rat muscle tissue, creatine supplementation has been reported to decrease the [PCr]/[TCr] ratio (5, 25). Ceddia and Sweeney (5) suggested that a decrease in the [PCr]/[TCr] ratio after creatine supplementation might cause the phosphorylation of AMPK. However, there is currently no published information regarding whether a change in the [PCr]/[Cr] ratio regulates rates of AMPK phosphorylation or dephosphorylation. Although there are reports of stimulation of AMPK phosphorylation independent of changes in concentrations of adenine nucleotides, creatine, or phosphocreatine (6, 10), the mechanism mediating the increase in AMPK phosphorylation by creatine supplementation in the present study and as previously reported (5) is unclear at this time.

MEF2 is a transcription factor that plays a key role in specific skeletal muscle gene expression (3). The activation of MEF2 in skeletal muscle is regulated via parallel intracellular signaling pathways in response to cellular stress or activation of AMPK (1). It has been demonstrated that the activation of AMPK increases MEF2 DNA binding activity (1, 32), resulting in increased muscle GLUT4 protein levels (16, 32). In our study, creatine feeding increased protein levels of MEF2A, -C, and -D isoforms in nuclear extracts of skeletal muscle. We also found that DNA binding activity of MEF2 was increased by 44% in creatine-fed rat EDL muscle nuclear extracts.

Some studies have not found increased GLUT4 after creatine feeding. For example, 5 days of creatine supplementation did not alter muscle GLUT4 content in rat skeletal muscle (17), and creatine supplementation did not affect muscle GLUT4 expression in a 6-wk supplementation period in humans (28). It has been proposed that creatine supplementation per se has no effect on either GLUT4 expression or glycogen content in humans but that it is effective in combination with exercise (8). However, it has been reported that creatine supplementation prevented a decrease in muscle GLUT4 protein content during 2 wk of immobilization (18), suggesting that creatine supplementation itself (without dietary supplementation or exercise) is able to affect muscle GLUT4 expression. At the present time, we are unable to explain the reasons for discrepant findings about creatine effects on GLUT4 expression. More studies need to be done to elucidate reasons for varying results on this issue and to clarify mechanisms.

In summary, we found that creatine feeding increases AMPK phosphorylation in muscle through an unknown process that does not require changes in creatine or phosphocreatine levels in muscle. Creatine feeding was related to enhanced nuclear content of MEF2, increased DNA binding activity of MEF2, increased in GLUT4 gene expression, and increased glycogen content in skeletal muscle. Our findings elucidate some of the possible physiological factors that might explain how creatine supplementation regulates GLUT4 biogenesis in muscle tissue.

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GRANTS

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Oral Creatine Supplementation and Athletic Performance: A Critical Review

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Objective: To review and summarize the current data on oral creatine supplementation regarding its potential efficacy in athletic performance, mechanism of action, and metabolism.

Data sources and study selection: Medline was searched using terms relating creatine supplementation to athletic performance. Studies that evaluated the effects of oral creatine supplementation on exercise performance in humans were selected for inclusion. Selected studies on muscle metabolism and exercise physiology were included if they provided useful information relative to creatine. Additional references were reviewed from the bibliographies of selected studies.

Data extraction and synthesis: To summarize efficacy, extracted data were listed in table format, grouping studies together by type of activity and efficacy on performance. Whenever possible, the effect of creatine supplementation was quantified. Proposed explanations for creatine's efficacy or lack thereof in a particular type of activity were formulated.

Conclusions: In laboratory settings, creatine supplementation is ergogenic in repeated 6–30-second bouts of maximal stationary cycling sprints. The data on a single sprint or first-bout sprint of any kind are inconsistent. The data regarding creatine's ergogenic effects on mass-dependent activities, such as running and swimming, are not convincing, perhaps because of the side effect of weight gain from water retention. Studies on weight lifting suggest that creatine improves strength, possibly by increasing myofibrillar protein synthesis; however, more study is needed to prove this. No ergogenic effects on submaximal or endurance exercise are evident. Individual response to creatine supplementation can vary greatly.

Key words: Creatine—Ergogenic—Nutrition—Supplements—Performance.

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In recent years there has been an unparalleled interest in the use of oral creatine by athletes and body builders hoping to increase strength and enhance athletic performance. Prevalent usage began after 1992, when Harris and colleagues (30) showed that oral creatine supplementation (20–25 g daily for 5–7 days) resulted in a 20% increase in skeletal muscle total creatine concentration. Although no claims could be made with regard to its effect on athletic performance, companies were quick to market the ergogenic potential of this legal substance.

Since the appearance of Harris' work, many subsequent studies have evaluated the potential ergogenic effect of oral creatine supplementation, and have met with varying results. The current critical review synthesizes the available data on oral creatine supplementation in order to allow conclusions about its potential applicability as an ergogenic aid. Data on creatine's metabolism and potential mechanisms of action are also discussed. We begin with a brief review of the role of creatine in energy metabolism of skeletal muscle.

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Creatine Metabolism

Synthesis/Exogenous Sources/Elimination

Creatine is an amino acid derivative naturally found in skeletal muscle (90–95% of creatine in the body is in skeletal muscle), cardiac muscle, brain, testes, and other organs (3,66). Of the creatine in skeletal muscle, approximately one third exists as free creatine, and two thirds as phosphocreatine (PCr) (3). Total creatine (TCr) is the sum of free creatine and PCr. Creatine is primarily synthesized by the liver, pancreas, and kidneys (3,41,65). Three amino acids (glycine, arginine, and methionine) and two enzymes are needed for endogenous creatine synthesis, which averages 1–2 grams each day (3,41,65). An additional 1–2 grams daily is obtained exogenously via dietary consumption of fish and meat (3) (Table 1). Creatine is eliminated by its irreversible conversion to creatinine at a rate of approximately 1–2 grams daily (65).

Transport

Creatine is transported into muscle tissue by a sodium-dependent transporter that has been cloned and expressed in cell systems (57), and whose expression is regulated by insulin (positive) and exogenous creatine (negative) (17,24,40).

TABLE 1. *Creatine content in uncooked* foods (3)*

Food	Creatine content (g/kg)
Herring	6.5–10
Pork	5.0
Beef	4.5
Salmon	4.5
Tuna	4.0
Cod	3.0
Milk	0.1
Cranberries	0.02
Shrimp	trace

* Some degradation of creatine occurs with cooking, though the extent is not known.

Muscle Concentrations

The average creatine concentration of human muscle is 125 mmol/kg dry mass (32), but individuals may range from 90 to 160 mmol/kg (26). Women may have slightly higher concentrations than men (22), and vegetarians tend to have lower concentrations than nonvegetarians (19).

Metabolism: Creatine/PCr as a "Temporal Energy Buffer"

During intense muscle contraction, ATP is rapidly depleted, as shown in the following equation:

$$ATP \xrightarrow{ATPases} ADP + P_i \quad (1)$$

Immediate regeneration of ATP is needed if muscle contraction is to continue. This ATP regeneration is dependent on re-phosphorylation of ADP, which is accomplished rapidly under *anaerobic* conditions via the following pathways (33):

$$PCr + ADP + H^+ \xrightarrow{CK} ATP + creatine \quad (2)$$

where CK is creatine kinase; and

$$Glucose/Glycogen + ADP \leftrightarrow ATP + lactate\ (anaerobic\ glycolysis) \quad (3)$$

PCr is a limiting factor in maintaining ATP resynthesis during maximal exercise (13,55). Specifically, 70% of PCr stores in Type II (fast twitch) muscle fibers are utilized after 10 seconds of maximal contraction, with near depletion after 20 seconds (33). The glycogenolysis/glycolytic pathway is already operating maximally within 1 second of maximal muscle contraction, and provides for proportionately more energy (ADP rephosphorylation) as contraction continues (33).

Metabolism: Creatine/PCr as a "Spatial Energy Buffer:" The Phosphocreatine Energy Shuttle

Creatine kinase (CK) has two isoforms in skeletal muscle: cytosolic and mitochondrial (miCK). These spatially separate isoforms catalyze the reversible reaction $PCr + ADP + H^+ \leftrightarrow ATP + creatine$, in their respective compartments (Fig. 1).

To elaborate, PCr stores are depleted after 10–20 seconds of intense exercise (33). The resynthesis of PCr requires ATP, which is generated aerobically by oxidative phosphorylation ($O_2 \rightarrow H_2O$) in the mitochondria (51). Once regenerated, this ATP is transported from mitochondria to the cytosol of skeletal muscle myofibrils, where it is utilized for the next bout of (predominately anaerobic) exercise. This shuttle system may also be important during aerobic exercise or in the facilitation of recovery after exercise. Thus, in normal subjects, the PCr shuttle acts as a "spatial energy buffer," providing for more efficient energy transport between sites of ATP synthesis and utilization (7,67).

Generally speaking, it takes 30–60 seconds to resynthesize half the PCr stores after a maximal bout of exercise, so most of the depleted PCr is restored within 5 minutes of recovery (3,56,61). It is important to remember that although the ATP synthesis from PCr is anaerobically derived, the resynthesis of PCr requires oxygen and is therefore aerobic.

Creatine Supplementation

Dosing

Creatine supplementation involves a loading dose of 20–25 grams daily for 5 days, followed by a "maintenance" dose of 2 grams daily (32). The loading regimen can increase total creatine stores by 17–22% (2,12,15, 21,23,27,30,32,46). Without a loading phase, a dose of 3 grams daily will achieve a similar increase after 28 days (32). The increase in total creatine is predominately due to a 2–40% increase in free creatine (12,23,32,50), although phosphocreatine (PCr) also increases, by 6–12% (12,23,30,32,63).

It has been shown that simultaneous consumption of carbohydrate with creatine supplementation further increases total creatine and PCr stores in human skeletal muscle *in vivo* (24). In addition, the magnitude of total creatine concentration increase is independent of the initial muscle creatine concentration (24). This is the basis of the commercially available combined creatine/carbohydrate preparations.

Muscle saturation and washout time of creatine

Skeletal muscle creatine has a saturation limit of 150–160 mmol/kg that cannot be exceeded by additional supplementation (3,26,30). This has important implications for those athletes who subscribe to the "more is better" philosophy and unnecessarily take higher doses. Hultman and colleagues (32) showed that 2 grams daily was enough to maintain the creatine stores achieved by the loading phase. If dosing by weight, the authors suggest a loading regimen of 0.3 g/kg daily for 5 days, followed by 0.03 g/kg daily as maintenance.

Muscle concentrations of creatine and PCr, as well as urinary creatine and creatinine concentrations, return to baseline levels approximately 28 days after discontinuing creatine supplementation (21,63). Because of individual variability, the "wash-out" time in future crossover studies should be at least 5 weeks.

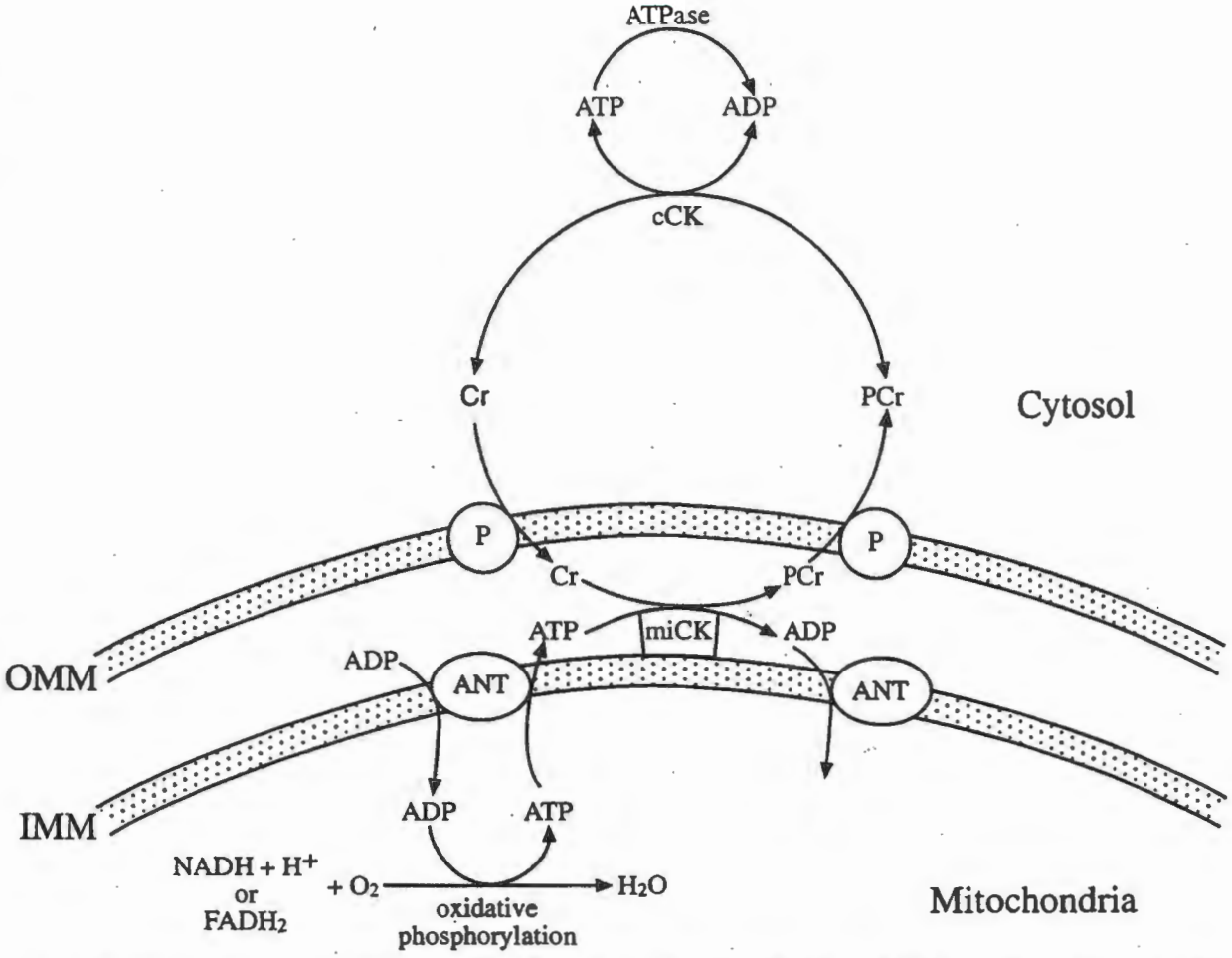

FIG. 1. The creatine/phosphocreatine energy shuttle (7,67). At rest, phosphocreatine (PCr) and creatine (Cr) exist in a reversible equilibrium in the cytosol (note reversibility of cytosolic creatine kinase [cCK] reaction). With contraction, PCr is needed to phosphorylate adenosine diphosphate to yield adenosine triphosphate (ATP). This ATP is then utilized to produce muscle work via the action of various ATPases. Implication of dreatine supplementation: creatine supplementation increases PCr stores, allowing for enhanced ATP production. To regenerate PCr during recovery after initial exercise, ATP is produced aerobically in the mitochondria via oxidative phosphorylation. This ATP is then transported across the inner mitochondrial membrane (IMM) via an adenine nucleotide translocator (ANT), and is then transphorylated by mitochondrial creatine kinase (miCK) to yield PCr. This PCr then passes through the outer mitochondrial membrane (OMM) via porin (P) and accumulates in the cytosol of skeletal muscle, ready for the next muscle contraction. Implication of creatine supplementation: the increase in cytosolic creatine stimulates the activity of miCK and mitochondrial oxidative phosphorylation, leading to enhanced resynthesis of PCr during recovery periods. ANT = adenine necleotide translocator; P = porin; NADH + H^+, $FADH_2$ = reduced nicotinamide and flavin (reducing equivalents).

Physiologic effects of creatine supplementation

Since creatine and PCr exist in equilibrium at rest (reaction 2) (26,51), creatine supplementation will increase PCr stores, which can theoretically prolong maximum power output.

In exercise longer than 20 seconds, however, anaerobic glycolysis (reaction 3) and aerobic mechanisms are the main contributors to ATP production, which may "dilute" any early benefit from the increased PCr (53). For example, in cycling of just 30 seconds duration, the aerobic contribution to energy production approaches 40–50% (9,42).

Creatine supplementation may also enhance the resynthesis of PCr during recovery periods, via stimulation of oxidative phosphorylation and the PCr shuttle (3,27). This enhanced aerobic recovery is the premise behind creatine's potential ergogenic applications in sports such as ice hockey and American football, which involve repeated bouts of predominately anaerobic maximal exercise interspersed with aerobic recovery periods.

Lactate

Blood lactate accumulation after high intensity exercise has been shown to be lower (1,2,4,48), the same (5 [protocol 2], 8,10–12,18,28,37,44,46,54,58,60,64 [protocol 1]), or greater (5 [protocol 1], 27,59,64 [protocol 2]) with creatine supplementation than in control subjects. A higher accumulation suggests an increase in anaerobic glycolysis for the generation of ATP, which could be a result of "activation" of glycolysis/glycogenolysis by PCr or free Cr (43). However, the numerous studies that demonstrate no significant difference in lactate accumulation suggest that oral creatine supplementation does not significantly increase anaerobic glycolytic flux in healthy humans.

Ammonia and Plasma Hypoxanthine

Several studies have demonstrated a decrease in ammonia or hypoxanthine accumulation in creatine supplemented subjects compared to placebo (1,2,4,8,28,44). This is probably a result of enhanced ADP phosphorylation by PCr, with a resultant decreased flux through adenylate kinase and myoadenylate deaminase. However, Snow and colleagues (54), in a double-blind cross-over study, did not demonstrate any difference in plasma ammonia or hypoxanthine accumulation among groups. Further cross-over studies are needed to determine whether Snow's results are reproduceable.

Oxygen uptake

Several studies demonstrated no change in oxygen uptake (VO_2) during high intensity (1,4,6) or endurance (1,5,58) exercise after creatine supplementation. This suggests that creatine supplementation does not enhance oxygen uptake *in vivo* in spite of its potential role in spatial energy buffering.

Creatine supplementation and body mass: Water retention, protein synthesis, and training stimulus

With few exceptions (29,49), a total mass increase has been shown to occur with creatine supplementation (2,4–6,15,18,20,27,44,54,64). An average of 0.5–1.6 kg is observed after just 5–7 days of creatine loading, with even greater amounts (1.8–2.42 kg) seen after longer periods of supplementation (39,63). Some studies have demonstrated that the increase is lean (fat-free) mass (20,39,63). Two explanations for this increase are water retention, which occurs rapidly on the first day of loading (32), and possible net nitrogen accretion, meaning enhanced myofibrillar protein/muscle synthesis, which may be seen with more prolonged use.

Increased water retention

Hultman and colleagues (32) demonstrated a significant (0.6 L) decrease in urinary output on the fist day of creatine loading (20 g daily) that normalized by the fifth day. This suggests that the initial body mass increase was a result of water retention, because 1–5 days would be too short a time for appreciable myofibrillar protein accretion to occur. However, increased water causes cell swelling, which may provide a molecular signal to increase muscle synthesis and/or decrease degradation (31). The implication is that longer periods of creatine supplementation result in enhanced myofibrillar protein synthesis.

Increase in myofibrillar protein synthetic rate

Two studies completed before creatine use became popular among athletes provide direct evidence of an increase in myofibrillar protein synthesis. Sipila and colleagues (52) demonstrated a 43% increase in the diameter of type II muscle fibers of the vastus lateralis after 1 year of creatine supplementation (1.5 g daily) in subjects who had gyrate atrophy (secondary creatine deficiency). More specifically, Ingwall and colleagues (34,35) demonstrated enhanced actin and myosin protein synthesis in skeletal muscle cultures when grown in a creatine medium as opposed to a creatine-free culture medium.

Creatine supplementation as a training stimulus

Creatine supplementation has been studied as a potential method of enhancing individual training. It has been suggested that the stimulus for increased myofibrillar synthetic rate is contraction *per se* (14,47). Therefore, if one can perform more contractions per unit of time, there may be a greater stimulus to increase muscle synthesis. Vandenberghe and colleagues (63) studied the effects of 10 weeks of creatine supplementation in sedentary females. One group (n = 10) ingested creatine during training, while the placebo group (n = 9) only trained. The creatine group showed an increase over placebo in maximal strength for leg press, squatting, and bench press. Fat-free mass also increased by an additional 1.0 kg for the creatine group compared with placebo (2.6 kg versus 1.6 kg).

In a similar study, Kreider and colleagues (39) studied 25 collegiate American football players during 28 days of resistance and agility training. The creatine group (n = 11) consumed 15.75 g of creatine during the 28 days. The creatine group demonstrated greater gains in fat/bone-free mass (2.43 kg gain versus 1.33 kg), bench press lifting volume, and stationary cycle sprint performance. However, there was no improvement in lifting volume for squats and power clean.

The above lends support to the hypothesis that creatine supplementation enhances the effects of training, possibly by enhancing protein synthesis/muscle accretion and/or allowing a greater total number of contractions over time. Whether this translates to better performance in an actual competitive event, however, is difficult to establish because of the increased variability in these outcome measures.

Studies of Potential Ergogenic Effect in Various Types of Exercise

Selection criteria

A 1990 to August 1998 MEDLINE search was performed, using the keywords "creatine" and "performance," "creatine" and "supplementation," and "creatine" and "strength." In general, selected studies were double-blind placebo-controlled trials with a minimum of 6 participants. Single-blind studies were not selected unless they employed a cross-over design. Studies published in abstract format were not selected. Using these criteria, 29 studies were reviewed and summarized. Only three (21,54,59) incorporated a cross-over design.

Studies

Single bouts of high intensity exercise (<60 seconds)

Most studies that evaluated a single burst of maximal exercise did not demonstrate an ergogenic effect of creatine. Four of these involved a single cycle ergometer sprint of 10, 15, 20, or 30 seconds (16,18,46,54) (Table 2). One study showed an ergogenic effect during 45 seconds of continuous jumping, yet no ergogenic effect during 5 seconds of continuous jumping (10). There are some studies involving multiple bouts of high intensity

TABLE 2. *Studies on single maximal cycling sprints (< 60 second duration)*

Author	Creatine dose	Subjects	Activity	Test	Results
Cooke 1995 (16)	20g Cr/4g gluc daily for 5 days, in water PL: 24g gluc daily	12 M, untrained (age 24.1, 81.2 kg)	Cycle ergometry	2 × 15-second max bouts (20 minute rest in between)	No improvement in peak power, time to peak power, total work, and fatigue index
Dawson 1995 (18)	25g Cr/1g gluc daily for 5 days, in warm water, tea, or coffee PL: 30g gluc daily	18 M, active (age 21.8, 77.1 kg)	Cycle ergometry	1 × 10-second max bout	No improvement in peak power, time to peak power, or work done
Odland 1994 (46)	20g daily for 3 days in flavored drink PL: drink only	9 M, physically active (age 22–30)	Cycle ergometry	1 × 30-second max bout	No improvement in peak power, mean 10-second power, mean 30-second power, or % fatigue
Snow* 1998 (54)	30g Cr/30g dextrose daily for 5 days PL: dextrose	8 M, active (age 23, 79 kg)	Cycle ergometry	1 × 20-second max bout	No improvement in peak power, time to peak power, or mean power

Cr = creatine monohydrate; PL = placebo; gluc = glucose; M = males; F = females
* cross-over study

exercise that demonstrated performance enhancement in the first bout (8,18,39,48). Other multiple-bout studies did not demonstrate a first bout performance enhancement (4,6,15,29,49).

Overall, the results on single sprints or first bout sprints must be considered inconsistent, making analysis difficult. As stated, the contribution of PCr to anaerobic ATP production diminishes with time, suggesting that longer bouts show an attenuated effect of creatine supplementation. However, there appears to be significant variability in the definition of a "longer" bout. In studies of single or first bout cycling sprints lasting 15 seconds or less, some demonstrated a benefit (18 [protocol 1],39), whereas others did not (4,6,15,16,18 [protocol 2]). In cycling bouts of 20–30 seconds, studies again were split between ergogenic (8,20) and nonergogenic (46,54) results.

Another factor to consider is that if a sprint is too short (e.g., 5–10 seconds), PCr stores may not deplete, even in subjects given placebo. This may explain the lack of ergogenic effect seen in studies involving 6–10 second cycling sprints (4,15,18), or 5 seconds of continuous jumping (10). However, Kreider and colleagues (39) did demonstrate an ergogenic effect in the first of multiple 6-second cycling bouts.

The inconsistencies outlined above suggest that there are unknown variables other than duration of exercise that affect the response to creatine supplementation, particularly if the exercise time is 30 seconds or less. However, few studies have demonstrated an ergogenic effect for exercise greater than 60 seconds, an observation that is addressed below.

Repeated bouts of maximal exercise

Most of the studies that demonstrated an ergogenic effect of creatine supplementation involved repeated maximal bouts of stationary cycling, specifically 6–30 second cycling bouts with 20 seconds to 5 minutes of rest between bouts (4,8,18,20,39,48) (Table 3). There are, however, several studies of this activity in which no ergogenic effects were demonstrated (6,15,21) (Table 4). In the studies that showed an ergogenic effect, the most common hypothesis was an enhanced resynthesis of PCr during the recovery periods. However, the studies in which no ergogenic effects were demonstrated warrant a closer analysis; they suggest that subjects respond differently to creatine supplementation. This will be addressed in detail below.

Weight lifting/strength

Several studies have demonstrated an ergogenic effect of creatine supplementation in weight lifting repetitions (Table 5) (20,39,63,64). Two of these also showed an increase in one-repetition maximum weight lifting, specifically, bench pressing (20) and leg press/leg extension (63). With respect to weight gain, Volek and colleagues (64) demonstrated a 1.4 kg increase in body mass during 7 days of supplementation, with no change in skinfold thickness. The increase, therefore, was believed to be a result of retained body water. Other studies (20,39,63) supplemented for longer periods of time (14 days to 10 weeks) and demonstrated 1.1–1.6 kg increases in fat-free mass versus placebo. Earnest and colleagues (20) found the increase in observed strength with creatine was still significant even when expressed relative to the increase in body mass. These studies support the hypothesis that creatine supplementation improves strength, and suggest that the observed fat-free mass increase is partially caused by net protein accretion. Other studies have demonstrated an ergogenic effect in specific muscle group activity, including knee extensions (28) and grip strength (59).

Submaximal exercise and maximal exercise of >60 seconds

Most of the data on submaximal or endurance exercise does not support an ergogenic effect of oral creatine supplementation (Table 6). This is probably because of the smallness of the role that the PCr energy system plays in muscle function during exercise of this nature (43,53,58), or because of increased weight with conse-

TABLE 3. *Studies on repeated maximal cycling sprints (< 60-second duration) demonstrating an ergogenic effect*

Author	Creatine dose	Subjects	Activity	Test	Results
Balsom 1993 (4)	25g Cr/5g gluc daily for 6 days PL: 30g gluc daily	16 M, phys ed students (age 26.7, 79.8 kg)	Cycle ergometry	A. 10 × 6-seconds (130 rev/minute), B. 10 × 6-seconds (140 rev/minute) 30 second recovery periods in both	A: no improvement in any bouts B: 4% increase in pedaling frequency vs. placebo during the 6-second intervals in bouts 7–10; No improvement in the first 6 bouts
Birch 1994 (8)	20g daily for 5 days in hot tea, coffee, or orange squash PL: gluc polymer	14 M, not highly trained (age 20.4, 70.9 kg)	Cycle ergometry	3 × 30-second bouts, 4-minute recovery periods	6% increase in mean power output in bouts 1, 2; no change in bout 3; 8% increase in peak power output in bout 1 only; 6% increase in total work performed in bouts 1 and 2, no increase in bout 3
Dawson 1995 (18)	25g Cr/1g gluc daily for 5 days, in warm water, tea, or coffee PL: 30g gluc daily	22 M, active (age 22.3, 75.5 kg)	Cycle ergometry	6 × 6-second bouts, 24-second recovery periods	4.6% increase in peak power bout 1; No increase in bouts 2–6; Total work increased 4.5%
Earnest 1995 (20)	25g daily for 14 days PL: gluc	8 M, weight-trained (age 30, 86.5 kg)	Cycle ergometry	3 × 30-second bouts, 5-minute recovery periods	13% increase in total anaerobic work in bout 1; 18% increase in bout 2; 18% increase in bout 3
Kreider 1998 (39)	15.75g Cr, 99g gluc, 3g taurine, 1.1g Na_2PO_4, 1,2 g KPO_4 daily for 28 days PL: same, but no Cr.	25 M, NCAA Div IA football players All subjects trained during the 28 days	Cycle ergometry	12 × 6-second bouts, 60-second recovery periods	Increase in total work during first 5 bouts
Prevost 1997 (48)	18.75g daily for 5 days, 2.25 g daily for 6 days PL: CaCl	10 M, 8 F physically active (age 23.5, 74.8 kg)	Cycle ergometry	Each done to exhaustion: A: continuous cycling B: 30-second cycle, 60-seconds rest C: 20-second cycle, 40 seconds rest D: 10-second cycle, 20 seconds rest	A: 23.5% longer work time versus pre-supp; B: 61.0% longer work time versus pre-supp; C: 61.9% longer work time versus pre-supp; D: >100% longer work time versus pre-supp

Cr = creatine monohydrate; PL = placebo; gluc = glucose; M = males; F = females

quently lower relative VO_2. Balsom and colleagues (5), in an analysis of performance in a 6-km terrain run lasting 20–26 minutes, reported a creatine-supplemented performance that was 26 seconds *slower* than unsupplemented performances, and believed weight gain to be the reason. Additional support for this idea came from the lack of improvement in a treadmill run to exhaustion lasting 3–6 minutes (5). Stroud and colleagues (58) found no change in respiratory variables in a 6-minute, 10 km/hour treadmill run at predetermined workloads from 50–90% of maximum VO_2. Tarnopolsky and colleagues (59) found no benefit in aerobic cycling performance and activities of daily living in seven patients with mitochondrial cytopathies (a disease of muscle that lowers PCr concentrations).

Rossiter and colleagues (50) demonstrated a 2-second improvement in a 1,000-meter rowing ergometer bout lasting approximately 3.5 minutes. They hypothesized that this was because of the 1:2 work:rest ratio of a rowing stroke, suggesting that the momentary rest period between individual strokes allowed for enhanced resynthesis of PCr in creatine supplemented subjects. How-

TABLE 4. *Studies on repeated maximal cycling sprints (< 60-second duration) demonstrating no ergogenic effect*

Author	Creatine dose	Subjects	Activity	Test	Results
Barnett 1996 (6)	20g Cr/20g gluc daily for 4 days PL: 40g gluc daily	17 M, recreationally active (age 20.5, 72.1 kg)	Cycle ergometry	7 × 10-second bouts, 30 seconds recovery between all bouts except between bouts 5 and 6 (5 minutes recovery)	No improvement in peak power output, mean power output, or VO_2 max all bouts
Cooke 1997 (15)	20g Cr/4g gluc daily for 5 days PL: 24g gluc daily	80 M, active (age 24.2, 81 kg)	Cycle ergometry	2 × 6-second bouts, w/ variable recovery periods of 30, 60, 90, and 120 seconds	No improvement in time to fatigue or peak power in any bouts
Febbraio* 1995 (21)	20g Cr in water daily for 5 days PL: 75g gluc daily	6 M, active age 23.8, 78.4 kg)	Cycle ergometry	4 × 60-second bouts, 1-minute recovery periods, then fifth bout to fatique	No improvement in time to exhaustion in bout 5, despite an increase in muscle total creatine concentration

Cr = creatine monohydrate; PL = placebo; gluc = glucose; M = males.
* cross-over study

TABLE 5. *Studies evaluating weight lifting or specific groups of muscle activity*

Author	Creatine dose	Subjects	Activity	Test	Results
Earnest 1995 (20)	25g daily for 28 days PL: gluc	8 M, weight-trained (age 30, 86.5 kg)	Weight lifting	1 max bench press, and max repetitions at 70% of max bench press until fatigue	6% increase in max bench press; 4 (26%) more reps at 70% max
Kreider 1998 (39)	15.75g Cr, 99g gluc, 3g taurine, 1.1g $diNaPO_4$, 1.2g KPO_4 daily for 28 days PL: same, but no Cr.	25 M, NCAA Div IA football players Subjects trained during the 28 days	Weight lifting	Max reps bench press (approx 4–8 lifts total) Max reps squat Max reps power clean	225 kg inc in total volume bench press; No increase vs PL in squats, power clean; 453 kg increase vs PL in total sum of bench press, squats, power clean
Vandenberghe 1997 (63)	20g daily for 4 days, then 5g daily for 10 weeks PL: maldextrin	19 F, sedentary (age 20.5, 59.1 kg) Subjects trained during the 10 weeks	Weight lifting	1-rep max leg press, leg ext, leg curls, squats, shoulder press; Intermittent arm flexion	20–25% increase vs. PL in 1-rep max of leg press, leg extension, and squat; Trend of greater strength in bench press and leg curls ($p<0.15$); No increase in shoulder press; 10–25% increase vs. PL in intermittent exercise capacity of arm flexors
Volek 1997 (64)	25g daily for 7 days PL: cellulose	14 M, resistance trained (age 24, 77.7 kg)	Weight lifting; Jump squats	A: 5 sets bench press to failure using subject's 10-rep max, 2 minute recovery periods; B: 5 sets of 10 reps jumping, at 30% of 1-rep max, 2 minute recovery periods	A: 1–2.3 more presses in all bouts; B: 50W (4%) inc in peak power all bouts
Andrews 1998 (1)	20g daily for 5 days PL: ?	20 M with CHF (age 63.5)	Muscle contraction	Handgrip contractions 5 s grip/5 s rest × 30 times, at 25%, 50%, and 75% max, then until exhaustion	No improvement at 25% max; No improvement at 50% max; Increased # contractions at 75% max
Gordon 1995 (23)	20g Cr w/gluc daily for 10 days PL: gluc	17 M with CHF Ejection fraction 29 ± 8% (age 43–70, 85 kg)	Muscle contraction	One-legged knee extensions, 60 contractions/minute Two-legged incremental exercise w/increase of 10 W/minute	No effect on cardiac ejection fraction; 10% increase in 2-legged performance; 21% increase in one-legged performance; 5% increase in peak torque
Greenhaff 1993 (28)	20g Cr/4g gluc daily for 5 days in warm tea or coffee PL: 24g gluc daily	12 M, active but not highly trained	Muscle contraction	5 bouts of 30 max knee extensions, 1 min recovery periods	5% increase in total peak torque in bouts 2,3,4, and during the final 10 contractions in bout 1, and during contractions 11–20 in bout 5
Tarnopolsky* 1997 (59)	10g daily for 2 weeks, then 4g daily for 1 week in juice, milk, or tea PL: glucose	3 M, 4 F with mitochondrial cytopathies (age 25–64, 57–88 kg)	Muscle contraction	Isometric hand gripping 9 seconds grip, 1-second rest cycle; Ankle dorsiflexion 15 seconds: 1-second work:rest × 2 minutes)	19% increase in grip strength; 11% increase in dorsiflexion torque

Cr = creatine monohydrate; PL = placebo; gluc = glucose; M = males; F = females
* cross-over study

ever, the magnitude of re-synthesis would be small, because the half-time PCr resynthesis rate is 30–60 seconds (61). Because stroke rate did not increase from pre to post-supplementation, the power per stroke may have increased, though this was not specifically measured.

In studies involving maximal exercise of "intermediate" duration, Terrillion and colleagues (60) found no benefit in creatine-supplemented subjects in two 700-meter running bouts (each run approximately 90–120 seconds). Jacobs and colleagues (36) demonstrated a 9% increase in time to exhaustion in creatine supplemented subjects during a cycle ergometer exercise at 125% of maximum VO_2 (approximately 135 seconds). Authors attributed this effect to a 10% increase in anaerobic energy, as measured by the maximum accumulated oxygen deficit (MAOD).

Interpreting the above data requires an understanding of the contributions of anaerobic versus aerobic energy to exercise. In submaximal or endurance exercise, the anaerobic component may be minimal, but there can be "spurts" of high-intensity anaerobic exercise (such as a "kick" at the end of a race). However, such energy is more likely to be derived from glycolysis than from PCr. Furthermore, longer bouts of exercise may "dilute" any benefit gained from short anaerobic spurts (53).

Overall, it appears that creatine supplementation does not enhance submaximal exercise or endurance exercise. This may indicate that the PCr shuttle function is not altered by creatine supplementation and/or the PCr shuttle is not rate-limiting in this type of exercise. Alternately, the mass gain may have an ergolytic effect, as outlined below.

Running and swimming: Weight gain as a possible hindrance in mass-dependent activity

It is interesting that most of the studies in which an ergogenic effect of creatine supplementation was demonstrated involved repeated stationary cycling sprints, isolated muscle contractions, or weight lifting, all of which are activities where body weight is supported.

TABLE 6. *Studies on submaximal exercise and maximal exercise > 60-second duration*

Author	Creatine dose	Subjects	Activity	Test	Results
Balsom 1993 (5)	20g Cr/4g gluc daily for 6 days PL: 24 g gluc daily	18 M, well trained (age 26.5, 73.6 kg)	Running-treadmill and terrain	Treadmill run to exhaustion (3–6 minutes) at 120% VO_2 max; 6 km terrain run (20–26 minutes) performed 1 day later	No change in treadmill run; 26 seconds (1.8%) slower in terrain run
Stroud 1994 (58)	20g daily for 5 days in hot drink No placebo	8 M, physically active (age 26, 76 kg)	Running-treadmill	10km/hour treadmill for 6-minute periods, from 50–90% of VO_2 max	No change in oxygen consumption (VO_2), no change in respiratory exchange rate, no change in lactate accumulation during exercise or up to 15 minute recovery
Bosco 1997 (10)	20g Cr w/gluc daily for 5 days PL: gluc	8 M athletes (age 20.5, 74.7 kg)	Running-treadmill	Run to exhaustion 20 km/hr, 5° inclination (approx. 1 minute)	13% improvement in time to exhaustion
Terrillion 1997 (60)	20g Cr/4g sucrose daily for 5 days PL: 24g suc daily	12 M, competitive runners (age 21.0, 70.2 kg)	Running-track	2 × 700 m, 60 min apart (each run approx. 90–120 seconds)	No improvement in running times
Jacobs 1995 (36)	20g Cr/1g gluc daily for 5 days PL: 3.6g gluc daily	21 M, 5 F recreational athletes (age 24.5, 78.3 kg)	Cycle ergometry	Cycle to exhaustion at 125% VO_2 max (130–140 seconds)	9% improvement in time to exhaustion (130–140 seconds); 10% increase in "maximum accumulated oxygen deficit"
Rossiter 1996 (50)	0.25g/kg daily for 5 days, in flavored drink PL: flavored drink	28 M, 10 F club standard oarsmen m: 22.7 yr, 78.1 kg f: 22.5 yr, 64.1 kg	Rowing ergometry	1,000m trial (approx. 210 seconds)	2.3 seconds (1%) mean improvement rowing time

Cr = creatine monohydrate; PL = placebo; gluc = glucose; M = males; F = females.

Studies of creatine supplementation on running and swimming are less convincing (Table 7), and it is possible that this could be a result of weight gain (5,44). Redondo and colleagues (49) failed to show an improvement in running velocity during three 60 meter running sprints, and Javierre and colleagues (37) found no improvement in a 150 meter running sprint. In contrast, Bosco and colleagues (10) demonstrated a 13% longer time to exhaustion in a treadmill run lasting approximately 1 minute. Three swimming sprint studies demonstrated no benefit (11,44,62), and one of them (44) demonstrated a tendency toward slower 25 meter and 50 meter swim times. Grindstaff and colleagues (29) showed an improvement over presupplementation performance in the second of three 100 meter swimming sprints, but no improvement in the first or third bouts. The effect of weight gain may also be detrimental to performance in endurance running, as shown by a study by Balsom and colleagues (5) in which creatine-supplemented subjects were 26 seconds slower than other subjects in a 6 kilometer run.

In light of the above data, the vague term "sprinting," often used by creatine advocates when describing its potential ergogenic effects, should be avoided, because it is clear that the majority of studies demonstrating an ergogenic effect involve stationary cycle ergometer sprints. Creatine's efficacy with regard to more mass-dependent activities, such as swimming and running, is inconclusive.

Critical Appraisal Factors

Subject to subject variability

The varying results of these studies strongly suggest that many factors influence the effects of oral creatine supplementation. These factors may include dosage amount and duration, exercise duration, fitness level of the subject, type of exercise performed, simultaneous carbohydrate ingestion, and length of recovery periods. However, one simple yet persuasive hypothesis is that subjects respond to creatine supplementation differently (25). In an often cited study, Greenhaff and colleagues (27) measured the resynthesis rate of PCr after intense electrically invoked muscle contraction. After results were obtained, the authors divided their eight subjects into two separate groups, labelling one group (n = 5) "responders," and the other group (n = 3) "nonresponders." Generally speaking, the responders had initially lower muscle creatine concentrations (<120 mm/kg), and greater percentage increases in total muscle creatine concentration (an average increase of 25%) after supplementation, as well as a faster rate of PCr resynthesis during the second minute of the recovery period. Nonresponders had only a 5% increase in total creatine concentration after supplementation and no improvement in PCr regeneration during recovery.

Harris in his initial work (30) did not specifically address PCr, but found that total muscle creatine concentration (PCr + free creatine) did increase by a greater percentage in those with initially lower total creatine concentrations, consistent with Greenhaff's work. Vegetarians, because of their initial lower concentrations of creatine (19), may benefit more from creatine supplementation, though this has not always been demonstrated (30).

The results from a cross-over study by Febbraio and colleagues (21), however, were not in support of Greenhaff's hypothesis. Their work included muscle biopsies to truly quantify the effect of supplementation on muscle

TABLE 7. *Studies on running sprints, swimming sprints, and jumping (greater mass-dependent activity)*

Author	Creatine dose	Subjects	Activity	Test	Results
Javierre 1997 (37)	25g daily for 3 days in flavored water PL: flavored water	12 national class sprinters	Running	150 m sprint	No improvement in running time
Redondo 1996 (49)	25g Cr/5g gluc daily for 7 days in tea or coffee PL: 25g gluc daily	8 M, 14 F, collegiate soccer or field hockey (age 20.6, 66.6 kg)	Running	3 × 60 m sprints (7–8 seconds each), 2-minute recovery periods.	No improvement in running velocity
Burke 1996 (11)	20g Cr/8g sucrose daily for 5 days PL: 20g polycose/ 8g suc daily	18 M, 14F elite swimmers	Swimming	25, 50, and 100 m sprints, 10-minute recovery periods;	No improvement at any distance
Mujika 1996 (44)	20g daily for 5 days PL: 20g lactose daily	11 M, 9 F elite swimmers (age 19.9, 70.3 kg)	Swimming	25, 50, and 100 m sprints 20–25-minute recovery periods	No improvement at any distance; Trend of slower times in 25 m and 50 m sprints.
Thompson 1996 (62)	2g daily × 6 wks w/water PL: ?	10 F, univ. swimmers (45.7 kg)	Swimming	1 × 100 m sprint, 1 × 400 m	No improvement in swim times
Grindstaff 1997 (29)	21g Cr/4.2g maldextrin daily for 9 days in water or juice PL: 25.2g maldextrin daily	7 M, 11 F nationally competitive juniors (age 15.3, 61 kg)	Swimming	3 × 100 m freestyle sprints, 60-second recovery periods	No improvement in Heat 1 and Heat 3; 0.93 seconds (1.4%) faster versus pre-supp in Heat 2
Bosco 1997 (10)	20g Cr w/gluc daily for 5 days PL: gluc	6 M athletes (age 20.5, 74.7 kg)	Jumping (for height)	Continuous jumping for 5 seconds Continuous jumping for 45 seconds	5-second jumping: No improvement avg power output; No improvement avg jump height 45-second jumping: 7% higher jumping ht in first 15 seconds; 15% higher in second 15 seconds; No improvement in third 15 seconds; No improvement in avg power output

Cr = creatine monohydrate; PL = placebo; gluc = glucose; M = males; F = females

creatine concentration. Activity involved four maximal 60-second bouts of stationary cycling with 60-second recovery periods, followed by a fifth bout of cycling to exhaustion. No ergogenic effect was observed, despite 6 of 7 subjects having initial muscle creatine concentrations lower than 120 mmol/kg, and all subjects significantly increasing their muscle total creatine concentrations.

One explanation for this discrepancy is the fact that Greenhaff and colleagues demonstrated that the rate of PCr resynthesis in the responder group was faster during the second minute of recovery and not the first minute (Febbraio's study used only 1-minute recovery periods). In addition, a 60-second exercise bout may involve a significant aerobic contribution to energy production. A similar cross-over design study involving predominately anaerobic 10–30-second bouts would be of interest.

Still, the responder/nonresponder hypothesis begged the consideration of studies to determine whether the extent of increase in PCr or total creatine concentration correlates with the extent of performance enhancement. In one study involving subjects with congestive heart failure (23), authors concluded a direct relationship between increase in PCr concentration and 1-legged cycling endurance and peak torque.

To summarize, initial muscle creatine concentration appears to be a contributing factor to the extent that muscle creatine and PCr concentration can be increased. The extent of this increase may correlate with increased performance, but other unknown factors are likely responsible. Future studies of "responders" and "nonresponders" must also identify the groups based on *a priori,* not *a posteriori,* measurement; otherwise, there is inherent bias.

Sample size and cross-over studies (Type II error)

None of the studies reviewed included a determination of sample size, and only three (21,54,59) incorporated a cross-over design. Larger sample sizes and cross-over designs reduce the chances of a type II statistical error (i.e., an acceptance of the null hypothesis when, in fact, it is false). Studies with smaller sample sizes, particularly without a cross-over design, need to be interpreted with caution. Given the small sample size in many of the studies investigating a potential ergogenic effect of creatine, it is possible that several studies that reported "negative" results had inadequate statistical power.

Effect of weight gain on double-blind studies

The fact that creatine supplementation results in weight gain brings into question the difficulty of effectively completing double-blind studies, as subjects who are aware they gain weight may incur a place bo effect. Specifically, knowledge of weight gain alone can

make a subject feel "stronger." Future studies should address this issue as objectively as possible.

Studies during actual competitive settings

Despite the number of studies demonstrating an improvement in stationary cycling and weight lifting, the fact that these studies were performed under controlled laboratory conditions rather than actual competition raises some doubt as to whether creatine supplementation truly enhances athletic performance. Most competitive timed events involve a single burst of activity, not repetitive bursts, and the data on single burst activity is inconsistent. Performance in sports that involve repetitive bursts, such as ice hockey and American football, is difficult to measure objectively. Furthermore, increasing muscle PCr concentration or lean body mass, whether a result of water gain or protein synthesis, does not unequivocally mean that athletic performance will be enhanced. It has been reported that up to 80% of the Olympic athletes in the 1996 Atlanta summer games used creatine at some point in their training, though no source for this information was given. If this is true, the most important question is what percentage of those athletes obtained a performance benefit during their actual Olympic event? Given the multiple factors that contribute to sports performance, the outcome measurements are inherently more variable than under laboratory conditions. Therefore, studies of sports performance should not be considered unless sample size estimates can demonstrate adequate statistical power (that is, avoidance of a type II error).

The elite athlete

An "elite" athlete can be defined as a senior national team member, Olympian, or professional. There is a striking paucity of data on oral creatine supplementation in these athletes, as noted by Mujika and Padilla (45) in their review. This could be relevant because of the high variability of response to creatine supplementation and the potential relationship between initial muscle creatine concentration and performance benefit. It is not known whether an "elite" athlete has higher initial muscle creatine stores than a sedentary individual. The only studies involving elite athletes have been in swimming (11,44) or running (37) and did not demonstrate an ergogenic effect of creatine supplementation. Until more data is obtained, no conclusions can be made regarding creatine supplementation's effects on this group of subjects.

Gender and age differences

Few studies have addressed gender differences in creatine supplementation. Women may have slightly higher total creatine concentrations relative to body weight than men (22). Rossiter and colleagues (50) demonstrated an increased uptake of creatine in men compared with women, but no conclusion could be made as to whether this influenced the results. No differences in results with respect to gender were noted in other studies that involved both sexes (11,29,36,44,48,49), though this question was not specifically addressed in the objectives of these studies.

In general, the process of aging results in a decrease of the free creatine/PCr ratio, which is likely a result of less activity in an older population (3). Total creatine stores relative to fat-free solids remain the same with aging, but decrease relative to alkali-soluble proteins (22).

Side Effects

The prevalent use of oral creatine by athletes has also created concern about potential side effects. Because of the extensive nature of this topic, the authors address it in a separate critical review (38).

Conclusions

Despite a significant amount of literature on oral creatine supplementation, there are deficiencies that need to be addressed. Caution must be exercised to avoid making statements that inherently expand creatine's ergogenic potential beyond what has been scientifically demonstrated.

Based on the evidence currently available, the following conclusions can be drawn:

- Oral creatine supplementation is ergogenic for repeated 6–30 second bouts of maximal stationary cycling, provided there are recovery periods of 20 seconds to 5 minutes. However, this has only been demonstrated under controlled laboratory conditions, and not all subjects respond.
- Oral creatine supplementation cannot be considered ergogenic for a *single bout* or *first-bout* sprint of any kind because the data are currently too inconsistent to allow such a conclusion.
- Oral creatine supplementation cannot be considered ergogenic for single or repeated swimming and running sprints (mass-dependent activity), probably because of the side effect of weight gain. However, the possibility of an ergogenic effect cannot yet be dismissed. To avoid confusion, the vague term "sprinting" should be avoided when discussing creatine supplementation, as the effect may be activity-dependent.
- Oral creatine supplementation may improve strength as a result of increased myofibrillar protein synthesis/muscle accretion, though this remains to be be proven.
- Creatine supplementation does not benefit submaximal or endurance exercise.
- Subjects with lower initial muscle creatine and PCr concentration will experience a greater percentage increase in total creatine and PCr concentration after creatine loading, which may correlate with increased performance.
- Creatine's ergogenic potential lies in enhanced anaerobic ATP production, enhanced resynthesis of PCr stores during recovery, and, possibly, enhanced myofibrillar protein synthesis/muscle accretion.
- Increase in lean body mass occurs with creatine supplementation. This increase is caused by water retention, and possibly by muscle accretion with longer use.
- There is insufficient data to formulate conclusions

about creatine's effect in actual competition (nonlaboratory) settings.

- Individual response to oral creatine supplementation can vary greatly in the extent of increase in muscle creatine concentration as well as performance results.
- Future studies, preferably using the cross-over design, should include larger numbers of subjects with determination of sample size. More studies outside the laboratory setting are needed, as well as studies on elite athletes. The interactive effect of weight gain on mass-dependent activity such as running and swimming should be further evaluated. Finally, studies are needed to establish the effect of creatine supplementation *per se* on muscle accretion.

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Popular Sports Supplements and Ergogenic Aids

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Abstract

This article reviews the evidence-based ergogenic potential and adverse effects of 14 of the most common products in use by recreational and elite athletes today. Both legal and prohibited products are discussed. This is an aggressively marketed and controversial area of sports medicine worldwide. It is therefore prudent for the clinician to be well versed in the more popular supplements and drugs reputed to be ergogenic in order to distinguish fact from fiction.

Antioxidants, proteins and amino acids are essential components of diet, but additional oral supplementation does not increase endurance or strength. Caffeine is ergogenic in certain aerobic activities. Creatine is ergogenic in repetitive anaerobic cycling sprints but not running or swimming. Ephedrine and pseudoephedrine may be ergogenic but have detrimental cardiovascular effects. Erythropoietin is ergogenic but increases the risk of thromboembolic events. β-Hydroxy-β-methylbutyrate has ergogenic potential in untrained individuals, but studies are needed on trained individuals. Human growth hormone and insulin growth factor-I decrease body fat and may increase lean muscle mass when given subcutaneously. Pyruvate is not ergogenic. The androgenic precursors androstenedione and dehydroepiandrosterone have not been shown to increase any parameters of strength and have potentially significant adverse effects. Anabolic

steroids increase protein synthesis and muscle mass but with many adverse effects, some irreversible. Supplement claims on labels of product content and efficacy can be inaccurate and misleading.

Products that claim to be performance enhancing are popular with recreational and elite athletes. Some are classified as drugs, others as supplements. Drugs that are used for medical purposes but have ergogenic properties are prohibited by most major sport governing bodies, since it is the elite athlete that is most likely to be tempted by such products. Supplements, however, are marketed aggressively to all types of athletes, which has generated its own controversy in this very profitable industry.[1]

Often, performance-enhancing products are purchased based on popular magazine advertisements, peer or coach recommendation rather than professional medical advice.[2,3] Furthermore, some products are popular and marketed as ergogenic despite a lack of objective evidence to support claims of an ergogenic effect. In the US for example, ergogenic claims of supplements can be made without verification by the Food and Drug Administration (FDA), ever since the controversial passage of the Dietary Supplement Health and Education Act in 1994.[4]

For this review, the Medline database was utilised to research the products listed (table I). Few studies had sample sizes of greater than 20 per group. Therefore, whenever possible, crossover designs were given high priority. Reviews of particular supplements or drugs were also analysed. Only reviews that provided a critique of studies rather than a rehashing of Medline abstracts were utilised. Many studies on over-the-counter (OTC) supplements were funded by supplement companies. While this did not necessarily exclude the study from consideration, conclusions from such studies were carefully assessed since supplements do not undergo the rigorous multiple-phase scrutiny of FDA approval. Surveys and case reports were judiciously utilised as some products have a paucity of controlled studies.

1. Antioxidants

An antioxidant is a product that can detoxify free radicals back into water and oxygen. Some antioxidants are endogenous to the human body, including glutathione and superoxide dismutase. The most popular antioxidants obtained by diet are ascorbic acid (vitamin C), tocopherol (vitamin E), coenzyme Q10, and β-carotene (precursor to vitamin A).[5]

A discussion of antioxidants must begin with defining a free radical, also known as a reactive oxygen species. A free radical is a molecule with an unpaired electron, and a normal by-product of life. Hydrogen peroxide (H_2O_2), superoxide (O_2^-) and hydroxyl radical (OH) are examples of free radicals. Free radicals are unstable, and produce cellular damage such as damage of lipid membranes and changes in membrane protein structure via lipid peroxidation.[6,7] The basis for antioxidants as a potential ergogenic aid lies in the fact that physical exercise increases oxygen uptake by body tissues resulting in oxidative stress, which leads to enhanced production of free radicals.[7] Free radicals can be a factor in prolonging recovery from exercise-induced fatigue.[5,7]

Antioxidant supplementation is popular among some endurance athletes, but the research does not support them as an effective ergogenic aid. In a double-blind, cross-over study, Nielsen and colleagues evaluated seven male triathletes taking the commonly marketed strategy of multiple antioxidants.[8] The athletes were supplemented for 6 weeks with 600mg ascorbic acid (reference daily intake [RDI] = 60mg), 270mg tocopherol (RDI = 20mg), and 100mg coenzyme Q10 (no RDI exists). No change was noted in maximal oxygen uptake ($\dot{V}O_{2max}$), muscle energy metabolism and muscle fatigue.

In another study of 25 blinded study participants (15 placebo and ten experimental), 2 weeks of 667mg (1000 IU)/day tocopherol supplementation

Table I. Popular sports supplements and ergogenic aids

Product	Ergogenic potential	Adverse effects/safety	Banned by	Typical dosage	Comments
Antioxidants	No	None known	None	Megadoses of ascorbic acid (vitamin C), tocopherol (vitamin E), β-carotene, etc.	Although not ergogenic, a diet high in antioxidants is recommended by most dietitians
Caffeine	Events >30 min = yes; 30 sec–30 min = probably; repetitive sprints = no	Dependency; withdrawal; CNS stimulant effects; mild diuretic (but exercise diminishes this effect)	IOC, FIFA: max. urine conc. of 12 μg/mL. NCAA: max. 15 μg/mL	2–9 g/kg (~150–700mg) PO 1h prior to event (8oz cup of coffee = 100–250mg, but coffee not ideal method)	Mechanism is adenosine receptor antagonism, and possibly enhanced fat metabolism which spares glycogen stores
Creatine	Repetitive max. cycling bouts = yes; running = no; swimming = no; repetitive bouts weight lifting = probably; isometric strength = no; endurance activity = no	Weight gain due to water retention; ↑ intra-compartment pressure in lower extremities – the likely cause of cramps; case reports regarding renal and muscle dysfunction	None	20 g/day PO for 5d; 2–5 g/day for maintenance; *or* starting with maintenance dose ok, although takes 28d to achieve what 5d of loading does	Ergogenic effect related to increased PCr stores that are converted to ATP; has not been shown to directly enhance protein/muscle synthesis; studies are difficult to do because individuals can often detect weight gain and know they are on creatine; weight gain may be detrimental to runners and swimmers; high individual variability
Ephedrine (ephedra, Ma Huang) and pseudoephedrine	Anaerobic activity = yes; endurance events = possibly	Hypertension; tachycardia; stroke; CNS over-stimulation	IOC, NCAA, NFL	Ephedrine: 1 mg/kg 1h prior to event. Pseudoephedrine: 60–180mg 1h prior to event	Mechanism due to CNS arousal as opposed to muscle metabolism. Pseudoephedrine is a drug. Ephedrine is marketed as an herbal supplement
r-HuEPO	Yes	Hypertension; thromboembolic events	IOC, FIFA, NCAA, NFL	50 IU/kg r-HuEPO IV or SC, typically for 15–30d	r-HuEPO extremely expensive, injectable only
HMB	Untrained individuals = possibly; trained individuals = no	Safe in studies of 8 wks or less, but few studies done	None	3 g/day PO	HMB is a metabolite of the essential amino acid leucine
HGH and IGF-1	Prescription injectable HGH can ↓ adipose tissue and ↑ lean body mass in the elderly. Performance studies lacking	Hypoglycaemia; ↓ endogenous HGH secretion; ↑ risk lung, colon cancer; TMJ discomfort; weight gain; dyspnoea; sinus tachycardia	IOC, FIFA, NCAA, NFL	For medical conditions, 6–12 μg/kg SC daily. Anecdotal reports that abusers use much higher doses	Beware the OTC products that are 'precursors', or homoeopathic amounts (600ng) of HGH, given sublingually or nasally (unproven methods of delivery). Real HGH is only available by Rx SC injection
Proteins, amino acids, branched-chain amino acids	No	Excess protein stored as fat, not utilised; theoretical concerns about renal load	None	Variable doses, PO	A proper diet is all that is needed, even for strength athletes, to get the protein they need. Protein and amino acid supplements have not been shown to enhance protein/muscle synthesis
Pyruvate	No	Unstudied	None	5 g/day	Never caught on – lack of ability to reproduce studies and lack of interest

Continued next page

Table I. Contd

Product	Ergogenic potential	Adverse effects/safety	Banned by	Typical dosage	Comments
Steroid precursor: androstenedione	No	↓ HDL; ↑ estradiol/estrogen; virilism	IOC, FIFA, NCAA, NFL, NBA[a]	Studies done on 100–300 mg/day PO. Product labels often instruct to take more	Although a testosterone/androgen precursor, does not promote protein/muscle synthesis. Can create positive drug test for testosterone
Steroid precursor: DHEA	No	↓ HDL; ↑ estradiol/estrogen	IOC, FIFA, NCAA, NFL, NBA[a]	25 mg/day PO, although studies up to 150 mg/day show no ergogenic effect	Although a testosterone/androgen precursor, does not promote protein/muscle synthesis. Can create positive drug test for testosterone
Anabolic steroids	Yes	Hirsutism; menstrual irregularities; aggression; ↓ spermatozoa; ↑ cardiovascular risk; liver dysfunction	IOC, FIFA, NCAA, NFL, NBA, MLB	250–3200 mg/week, PO or injectable. Many alternate dosage strategies exist.	Enhances protein/muscle synthesis

a The NBA Players Association has filed a grievance challenging the banning of androstenedione and DHEA. The National Hockey League does not keep a list of banned substances and only ban substances in accordance with local laws; however, this is under constant scrutiny for potential change.

ATP = adenosine triphosphate; **conc.** = concentration; **DHEA** = dehydro-epiandrosterone; **FIFA** = Fédération Internationale de Football Association (Soccer); **HDL** = high-density lipoprotein; **HGH** = human growth hormone; **HMB** = β-hydroxy-β-methylbutyrate; **IGF-1** = insulin growth factor-1; **IOC** = International Olympic Committee; **IV** = intravenously; **max.** = maximum; **MLB** = Major League Baseball; **NBA** = National Basketball Association; **NCAA** = National Collegiate Athletic Association (USA); **NFL** = National Football League (American Football); **OTC** = over-the-counter; **PCr** = phosphocreatine; **PO** = orally; **r-HuEPO** = recombinant erythropoietin; **Rx** = prescription; **SC** = subcutaneously; **TMJ** = temporomandibular joint; ↑ = increase; ↓ = decrease.

did not enhance performance in a marathon run.[9] Coenzyme Q10 for 28 days did not affect $\dot{V}O_{2max}$, heart rate, blood pressure, or anaerobic respiratory threshold in a study of male cyclists and triathletes (eight experimental, ten placebo).[10] A large dose of ascorbic acid (400 mg/day) for 2 weeks did not improve muscle soreness, muscle damage (measured by creatine kinase), or lipid peroxidation (measured by malondialdehyde) in active male runners.[11] It must be noted that most of these studies involved short-term supplementation, and the results may be different with long-term supplementation. Additionally, small sample sizes not performed with a crossover design increases the likelihood of a Type II statistical error, meaning an ergogenic effect may exist, but the sample size is too small to see it. Further research into antioxidants with larger sample sizes or crossover designs would help clarify the ergogenic potential of antioxidants.

It is important to distinguish an ergogenic effect from an objective measurement of cellular damage. Several studies have shown that supplementation with antioxidants can improve measurements of oxidative stress in humans.[6,12] Such measurements are very complex, but generally involve by-products of lipid peroxidation (conjugated dienes, thiobarbituric acid-reactive substances, malondialdehyde, or lipid peroxides). While such results have potential clinical implication in preventive healthcare, they should not be interpreted as proving ergogenic potential.

Regardless of ergogenic potential, it is well accepted that antioxidants are a key dietary component for athletes and non-athletes alike. In those athletes who restrict dietary intake, particularly if they lack in fruits and vegetables, supplementation with antioxidants is advisable. The potential for antioxidants to prevent muscle damage and lipid peroxidation resulting from high level repetitive training warrants further study, as such prevention may have long-term health benefits.[6,13] Antioxidants may not be truly ergogenic and supplementation is not necessary in most cases; however, athletes are still advised to ingest a diet rich in antioxidants.[5-7]

2. Caffeine

Caffeine is an adenosine-receptor antagonist and a stimulant of the dimethylxanthine class. Caffeine can be considered a drug as it is an ingredient in some pharmaceutical products, but since it is so ubiquitous OTC, it is often classified as a supplement. The mechanism for the ergogenic effect of caffeine remains somewhat inconclusive in humans, although rat studies clearly show that caffeine inhibits adenosine receptors,[14] and such receptors are located throughout the human body. On the cellular level, a recent rat study found that the phosphorylation and dephosphorylation of dopamine- and cyclic adenosine monophosphate (cAMP)-regulated phosphoprotein of relative molecular mass 32 000 (DARPP-32) plays a role in the stimulant action of caffeine.[15] Another theory put forward is stimulation of adrenaline secretion, resulting in the mobilisation of free fatty acids – an important fuel for muscle. This increase in fat utilisation decreases carbohydrate utilisation, thus delaying glycogen depletion.[16] While there have been studies to support this latter theory,[17] recent studies have failed to support it.[18,19]

The strength of evidence of caffeine's ergogenic potential is strong, particularly in aerobic activity.[16,20] In a double-blind, crossover study, Kovacs et al. studied 15 well-trained male triathletes and cyclists in a 1-hour cycling time trial at 75% of work maximum.[18] Three different dosages of caffeine were consumed: 154mg (2.1 mg/kg), 230mg (3.2 mg/kg) and 328mg (4.5 mg/kg). Even in the lowest dosage, there was improvement in time trial performance. Interestingly, the highest dosage was no more efficacious than the middle dosage, indicating a possible saturation effect of caffeine as an ergogenic aid. Urinary concentrations of caffeine were also measured (samples collected 5 minutes post-exercise), and did not reach any higher than 2.5 μg/mL. This is well below the disqualification limits imposed by the International Olympic Committee (IOC) [12 μg/mL] and the US-based National Collegiate Athletic Association (NCAA) [15 μg/mL].

In a double-blind, crossover study involving physical activity of a shorter duration, Bruce et al. found enhancement of 2000m rowing performance after caffeine consumption of either 6 or 9 mg/kg.[21] The improvement in time averaged 1%, with a 3% improvement in power output when comparing both caffeine groups to placebo. Similar to Kovacs study,[18] the higher dose of caffeine was no more efficacious than the lower dose. However, urinary concentration at the higher dose did reach as high as 14 μg/mL, a disqualifying number based on IOC standards. One strength of Bruce's study is that all eight study participants were well trained.

Even for sprint activity, caffeine has shown ergogenic promise in a single cycling[22] or swimming[23] sprint. However, Paton et al.[24] demonstrated in a double-blind, crossover study that caffeine is not ergogenic for repetitive bouts of sprinting, which is commonly used in team sport workouts.

Generally speaking, the doses of caffeine used in studies range from 2–9 mg/kg (about 250–700mg caffeine), taken 1 hour or less prior to the event. Clearly, an ergogenic effect of caffeine in aerobic activity is demonstrated in doses that would not reach the disqualifying levels of NCAA and IOC sport, as it takes approximately 9 mg/kg consumed to achieve a urinary concentration of 12 μg/mL.[18]

Adverse effects of caffeine are minimal; however, its central nervous system effects can cause anxiety, dependency and withdrawal. The diuretic effect of caffeine, although theoretically a concern in endurance events, appears to be attenuated or eliminated by exercise.[18,25]

The use of caffeine by athletes is not surprisingly common. Caffeine does not have the stigma branded to it that some supplements do, and is quite safe. It is likely that many Olympians use caffeine since the cut-off for disqualification is considered high enough to warrant taking the risk. Athlete beware, however, that urinary measurements of caffeine concentration are notoriously inaccurate.[18]

Finally, although coffee is not considered the ideal method of consumption of caffeine (tablets are the usual recommendation), it should be emphasised that coffee can vary greatly in its caffeine content. For example, an 8oz cup of brewed Maxwell

House®[1] coffee contains 110mg, and an 8oz Starbucks® cup of coffee has 250mg.[26] Caffeine content in common foods and drink are summarised by Harland.[26]

3. Creatine

Creatine is a nitrogenous compound that exists naturally in skeletal muscle in an equilibrium with phosphocreatine (PCr). PCr is the primary source of adensosine triphosphate (ATP) in skeletal muscle during intense, burst-type (anaerobic) exercise.[27] Creatine supplementation allegedly exerts its ergogenic effect by increasing resting concentrations of creatine in skeletal muscle, which subsequently increases PCr concentration by 12–18%.[28] This allows for more ATP availability, which is rapidly used during intense exercise.[29] Additionally, creatine causes a rapid weight gain due to water retention,[27,28,30] and it has been hypothesised that intracellular water retention may stimulate protein/muscle synthesis.[31] However, despite manufacturer claims, creatine itself has not been shown to be anabolic (i.e. it does not enhance protein synthesis and therefore does not increase muscle mass).[29,32,33]

The typical regimen involves oral intake of 20 g/day for 5 days as a loading dose, followed by 5 g/day as a maintenance dose. However, athletes often take more than is needed,[3,34,35] and even the 5g maintenance dose is more than necessary, as 2 g/day is enough to maintain the maximum elevated creatine concentration in skeletal muscle.[30] A study by Green et al.[36] found that carbohydrate ingestion enhanced performance over creatine alone, allegedly by enhancing the insulin-dependent creatine transporter to increase muscle uptake of creatine. However, such doses of carbohydrate are known to cause their own kinds of problems such as diminished gastric motility which can adversely affect performance.

No sports supplement has been studied more than creatine, but there remains substantial controversy over its alleged ergogenic effect due to the numerous 'ergogenic' studies with questionable methodology and biased conclusions.[28,29,37,38] For example, authors sometimes conclude an ergogenic effect in the highly visible abstract that appears on the Medline database, but a detailed analysis of the body of the paper raises questions as to the validity of such conclusions.

A recent double-blind, crossover study with excellent methodology did find an ergogenic effect of creatine in repetitive bouts of high-intensity cycling and a maximal voluntary dorsiflexion of the ankle.[39] However, creatine has not been shown to enhance all strength parameters. It has not been shown to enhance isometric strength[40] or overhead motion strength.[41,42] There are seemingly countless ways to evaluate muscle strength, including peak torque, maximal voluntary contraction, peak power, and one repetition maximum. Since many athletic endeavours involve various muscle activities, the average clinician can find such strength measurements confusing and difficult to clinically correlate.

There is considerable scepticism as to the ergogenic potential of creatine in mass-dependent sports such as running and swimming. This is because consumption of creatine results in a rapid weight gain due to intra- and extra-cellular water retention,[27-30] which can be detrimental to running or swimming performance. The great majority of 'sprinting' studies are on stationary cycles in laboratories, which is not the same as a running or swimming sprint. In an endurance run of 6km, creatine-supplemented individuals actually ran 26 seconds slower than the placebo-supplemented individuals.[43] There have also been several studies reviewed[28] that demonstrated no benefit in running sprints and swimming sprints. A recent study, however, did conclude an ergogenic effect of creatine in repetitive 5m and 15m running sprints.[44] The study participants were well-trained soccer players and the study was designed to be more akin to a true field test than the many previous laboratory studies; however, the experimental group was small (n = 8).

A more recent running study by Cox et al.[45] demonstrates how difficult it is to interpret many of

1 The use of tradenames is for product identification purposes only and does not imply endorsement.

the creatine studies that exist today. In one of the few on-field studies on creatine, Cox et al.[45] studied 12 female soccer players (six experimental, six placebo) in 20m running sprints and agility runs. A total of 55 running sprints and ten agility runs were studied. In nine of the 55 sprints, and three of the ten agility runs, the creatine group achieved faster post-supplementation times. However, this means that most individuals taking creatine did not improve their times (46 out of 55 sprints, seven out of ten agility runs). Additionally, in all study participants, there was no change in overall sprint time nor overall agility run time. Through no fault of the authors, such a study is often interpreted by the athletic community in a very 'ergogenic' light.

The simplest conclusion regarding creatine is that it is ergogenic for repetitive bouts of high intensity anaerobic exercise which are not mass-dependent (such as cycling), and for certain parameters of strength including one-repetition maximal voluntary contraction. However, its effect is highly variable between individuals. The weight gain associated with creatine is likely the reason why an ergogenic effect in running and swimming is questionable, probably more so with swimmers due to an increased drag effect.[46] It should be noted that the rapid weight gain from creatine supplementation also makes it very difficult to blind individuals in such studies.

There are two proven adverse effects of creatine. One is weight gain, which has been well documented,[27,47] and the other is increased muscle compartment pressure.[48] This increase is likely the reason for the numerous reports of muscle cramping in athletes who take creatine.[3,27,34,49] Recently, Robinson reported a case of rhabdomyolysis in an individual taking creatine.[50] While a cause and effect relationship cannot be firmly established, the data of Schroeder et al.[48] and the numerous reports of muscle dysfunction should make the clinician wary that creatine is not entirely innocuous, and any athlete taking creatine should report muscle pain or cramping to a physician.

Renal parameters such as glomerular filtration rate and serum creatinine levels can be affected by creatine, but in studies of 10 weeks or less such effects were reversible.[47] An animal study found progression of renal disease as a result of creatine supplementation,[51] and there have been two published reports of renal dysfunction in humans taking creatine.[52,53] However, both individuals were taking more than the recommended dose, and one[53] had previous renal disease. Nonetheless, since skeletal muscle has a threshold of how much creatine it can store, patients should be informed that the 'more is better' philosophy does not apply with creatine, and only increases chances of adverse effects.

Finally, it should be noted that creatine is naturally found in almost all tissues, including testes, liver, kidneys, heart and brain. Creatine supplementation increases brain creatine levels in humans,[54] and animal studies demonstrate increased creatine levels in kidneys, lungs and liver after oral supplementation.[55] The significance of these findings is unknown.

Patients who insist on taking creatine should be told to dose appropriately, be aware that creatine may or may not work for them, and that creatine is taken at their own risk.

4. Ephedrine (Ephedra) and Pseudoephedrine

Ephedrine and pseudoephedrine are sympathomimetic amines known for their stimulant properties. For this reason, many athletes use them in the hope of increasing energy and delaying fatigue. Ephedrine, also classified as ephedra alkaloids, is classified as an herb, and therefore a dietary supplement. Pseudoephedrine is classified as a drug, and is the world's most commonly used decongestant sold OTC. Both are also marketed as appetite suppressants used for weight loss, leading to indiscriminate use by wrestlers wishing to reduce body mass prior to competition.

It has been proposed that sympathomimetic agents such as ephedrine and pseudoephedrine are ergogenic by a glycogen-sparing mechanism, but this has not been supported in recent work.[56] Ephedrine increases the release of monoamines such as dopamine both centrally and peripherally.[57] This

may explain its ergogenic effect in some studies but again remains only a theory.

In a study of 16 male participants, Bell et al.[58] found that 1 mg/kg ephedrine improved anaerobic performance in a 30-second Wingate test, although the study participants were untrained. The authors hypothesised the ergogenic effect to be due to increased arousal as opposed to enhanced muscle metabolism. Bell et al.[56] also studied 12 individuals (ten men and two women, all recreational runners) in a 10km run, and found that 0.8 mg/kg ephedrine improved running time from 46.8 ± 3.2 minutes to 45.5 ± 2.9 minutes, suggesting that ephedrine is ergogenic in prolonged exercise. Thus far, no published studies have evaluated ephedrine in burst-type activity.

Most studies on pseudoephedrine have not demonstrated an ergogenic effect.[59-62] Two randomised, double-blind, crossover studies involving ten individuals each demonstrated no ergogenic effect of 120mg pseudoephedrine.[59,60] One study involved a 1-hour bout of high-intensity cycling,[59] the other involved maximal anaerobic output and fatigue.[60] Even high-dose pseudoephedrine (240 mg/day for 3 days) did not enhance performance of eight trained male runners in a 5000m timed treadmill run at 70% $\dot{V}O_{2max}$.[63] However, Gill et al.[61] did a double-blind, crossover study of 22 male athletes who took 180mg of pseudoephedrine. There was an increase in maximum torque in an isometric knee extension, increase peak power in cycling, and improved forced expiratory volume in 1 second. Taken together, the above studies suggest that pseudoephedrine is not ergogenic in aerobic or endurance activity, but at a high enough dose, may be ergogenic in maximal anaerobic burst-type activity.

Adverse effects of pseudoephedrine and ephedrine are of great concern, primarily because of the high incidence of cardiovascular events reported. Since pseudoephedrine is a drug, adverse effects are well documented as with other sympathomimetic drugs. Label precautions centre on cardiovascular and central nervous system effects.[64]

Recent high-profile deaths of athletes taking ephedrine supplements have led to an FDA consumer warning in the US.[65] A detailed review by Bent et al.[66] found that ephedra-containing products accounted for 64% of all adverse reactions to herbs in the US, despite representing only 0.82% of sales. Higher doses of ephedra also increases the risk of haemorrhagic stroke.[67] A detailed 22-month review[68] found 140 adverse events related to the use of supplements containing ephedra alkaloids, primarily involving cardiovascular events and stroke. Ten involved death, and 13 resulted in permanent disability. Dosages of ephedra alkaloids in most of these cases ranged from 20–60 mg/day, clearly within range of many OTC products.

In summary, sympathomimetics such as ephedrine and pseudoephedrine may differ in their ergogenic potential. Pseudoephedrine may have ergogenic potential in burst type activity but not prolonged activity. Ephedrine has potential in activity of longer duration, but the mechanisms are unclear. Intuitively, sympathomimetics may be ergogenic due to a stimulant effect, but safety reasons alone should place them on the not-recommended list, and they are banned by most sport governing bodies.

5. Erythropoietin

Erythropoietin (EPO) is produced naturally by the kidneys to regulate red blood cell production,[69] and is approved for medical use in chronic renal failure and certain types of anaemia. It remains, unfortunately, a widely abused drug in the sporting world, particularly with the advent of recombinant human EPO (r-HuEPO), which allowed for convenient subcutaneous injection as opposed to autologous blood doping in the older days.[70] Darbepoetin (Aranesp®), a newer product approved for chronic renal failure, is the newest agent. Its detection resulted in the disqualification of three cross-country skiers, including a gold and silver medal winner, in the 2002 Salt Lake City Winter Olympic Games. Darbepoetin has a 3-fold longer terminal half-life than r-HuEPO (21 hours intravenously, 49 hours subcutaneously), and therefore does not have to be administered as often as r-HuEPO.[71] For example, in chronic renal failure, if a patient uses r-HuEPO 2–3

times weekly, the frequency of darbepoetin is only once weekly.

There is no argument that EPO is ergogenic due to its ability to improve the oxygen carrying capacity of blood.[72-74] Audran et al.[72] studied the effects of subcutaneous injection of 50 IU/kg of r-HuEPO for 26 days in nine athletes (seven men and two women). Haemoglobin and haematocrit values were significantly elevated by the end of treatment. Each athlete performed an incremental exercise cycle test to exhaustion. $\dot{V}O_{2max}$ improved by 9%, power output by 7%, and maximum heart rate decreased by 5%.

EPO is an unquestionably dangerous substance for athletes to use. Potential adverse effects centre on thromboembolic events due to the increased packed red blood cell count and viscosity after administration. The most common adverse effects seen with medical use of EPO are hypertension, seizures and thromboembolic events.[75,76] There is little doubt in the minds of many in the medical community that several untimely deaths of elite cyclists and other athletes were related to blood doping or the use of exogenous EPO.

Advances have been made in the detection of autologous or recombinant EPO administration, as it is banned by most major sporting organisations. A 50% haematocrit rule was imposed by the Union Cycliste Internationale, and an 18.5 g/dL haemoglobin limit by the Federation Internationale de Ski. However, these numbers have been subject to criticism because of the effects on such parameters due to posture, dehydration and exercise.[77,78] Additionally, a small percentage of athletes have such values as baseline,[77] and high altitude living elevates baseline values.[79]

Detection of EPO abuse has been implemented at major sporting events such as the Tour de France and the Olympic Games.[1] Detection tests centre on the fact that r-HuEPO administration causes a predictable haematological response involving haematocrit, serum erythropoietin, reticulocyte count, macrocyte count, and soluble transferrin receptor. Parisotto and colleagues have revealed some promising and reproducible results in two consecutive studies,[77,80] which are much more sophisticated than the crude measurement of one haemoglobin/haematocrit value. They concluded that r-HuEPO administration not only causes such predictable and reproducible haematological responses, but they exist even several weeks following r-HuEPO administration. Furthermore, results did not vary with ethnicity. Continued research into detection methods will undoubtedly yield testing that is superior to an arbitrary limit of haemoglobin or haematocrit.

Nonetheless, the administration of sophisticated tests faces a major hurdle, as their cost can be prohibitive. Recently, the 'GH2000' project, which was an international effort to detect abuse of growth hormone, was dropped due to lack of IOC funding, even though it was acknowledged that better detection is needed.[1] It is easy to see why the perpetrators are often one step ahead of the detectors.

6. β-Hydroxy-β-Methylbutyrate

β-hydroxy-β-methylbutyrate (HMB) is a metabolite of the essential amino acid leucine. It is promoted as a supplement to increase strength and lean body mass not by being truly anabolic, but rather anti-catabolic (preventing muscle breakdown).

HMB is metabolised to hydroxymethylglutaryl-coenzyme A, which has been hypothesised to be the rate-limiting enzyme when cholesterol synthesis is in demand.[81] Because cholesterol synthesis is needed in membrane repair, it is thought that HMB supplementation can decrease muscle damage and enhance recovery. Despite the popularity and heavy marketing of this hypothesis, it has yet to be proven, and studies on the ergogenic effect of HMB have been equivocal.[82]

Knitter et al.[83] studied 13 untrained individuals supplemented with 3 g/day of HMB for 6 weeks, and found that the HMB group had a lower increase in creatine phosphokinase and lactate dehydrogenase after a 20km run, supporting the hypothesis that HMB may prevent muscle damage. However, the study does not address performance, and does not support, or refute, the cholesterol synthesis theory.

In a study funded by two sports supplement companies, Gallagher and colleagues[84] studied un-

trained college men during 8 weeks of resistance training, supplementing one experimental group (n = 12) with 3 g/day of HMB, the other group (n = 11) with 6 g/day HMB. The results were mixed and therefore difficult to draw conclusions from. One repetition maximum strength did not improve in either group. The 3 g/day group did show a greater increase in peak isometric torque than placebo, but interestingly, greater than the 6 g/day group also. No differences were observed in body fat between the three groups, but the 3 g/day group showed a greater increase in fat-free mass than placebo. Strangely, the 6 g/day group did not show a greater increase in fat-free mass than placebo. The authors conclude that HMB appears to increase peak isometric and isokinetic torque values, and increase fat free mass. Such a conclusion seems a bit tenuous since the two experimental groups varied in their results.

Equivocal results were also obtained in a supplement company-sponsored study by Panton et al., where 21 individuals supplemented with 3 g/day HMB for 4 weeks demonstrated an increase in upper body strength compared with placebo.[85] However, lower body strength did not improve. In another study on untrained individuals, Jowko et al. studied 3 g/day HMB supplementation with resistance training in nine men, and found beneficial results in six of seven strength tests compared with placebo, but no significant changes in body fat or lean body mass.[86]

The results of HMB on trained individuals are less promising. A recent study by Slater and colleagues found that HMB supplementation for 6 weeks did not affect strength or body competition in trained men.[87] It is thought that this may be due to the training-induced suppression of protein breakdown.[88]

On a positive note, Gallagher and colleagues found that HMB did not adversely affect lipid profiles, hepatic enzyme levels or renal function in their study participants.[89] The safety of HMB was further addressed by Nissen and colleagues at Iowa State University and no untoward effects were seen, although the longest study was 8 weeks in duration.[90]

HMB, while probably safe for 8 weeks or less, may have a role as an ergogenic aid in untrained individuals; however, its data on trained individuals is less convincing. Further research, not only on performance but also the cholesterol synthesis hypothesis, would be of value in understanding the role of HMB in exercise and muscle breakdown.

7. Human Growth Hormone and Insulin-Like Growth Factor-1

Human growth hormone (HGH) is synthesised by the anterior pituitary gland, and its metabolic effects are mediated by the hormone insulin-like growth factor-1 (IGF-1).[91] HGH is a prescription drug that has been shown in men over 60 years of age to increase lean body mass, decrease adipose tissue, and slow the thinning of the skin.[92] It is therefore classified as 'anti-aging' by some, a rather dubious terminology. Not surprisingly then, there has been recent interest in the use of HGH (also abbreviated GH) as a sports supplement[93,94] even though it remains essentially unstudied in younger populations. The postulated mechanism behind HGH in athletes includes enhanced amino acid and glucose uptake in skeletal muscle, thus stimulating protein synthesis, possibly combined with increase of free fatty acid mobilisation as an energy source.[93] HGH also stimulates IGF-1 synthesis, which is discussed later in this section. The strong endorsement bestowed upon HGH in the notorious publication *'The Underground Steroid Handbook'* helped solidify the place of HGH in the elite athlete's mind, despite no evidence to support the aforementioned theories.

What is often misunderstood, however, is that HGH is a drug only available by injection, and is not available OTC or on the Internet. The HGH molecule is too big to be absorbed in the gastrointestinal tract, and is broken up into its constituent amino acids when taken orally.[95] OTC products are labelled precursors, secretagogues or releasers of HGH. There is no evidence to suggest that such precursors of HGH are effective as an ergogenic, weight-loss or anti-aging agent. Advertisers of these products often cite Rudman et al.'s study,[92] without

mentioning that the study used real, injectable, prescription HGH. Furthermore, the participants in the study were men over 60 years of age, who naturally would have lower GH levels to begin with. No legitimate published studies on OTC products have been done, and therefore their safety and efficacy is in question.

Recently, some advertisers claim to have real HGH in their product. The concentration of HGH within these products (usually 600ng) are not only minute and unverified by the FDA, but are also derived from different sources than prescription HGH. Furthermore, the claimed superior method of delivery (nasal or sublingual) is speculative at best. Again, safety is not established, and there is legitimate concern about black market HGH that is pituitary (not recombinant) derived, which runs the risk of Creutzfeld-Jacob disease or other contamination.[95]

Certainly, prescription HGH has legitimate medical uses, including Turner syndrome in children, and Adult Growth Hormone Deficiency syndrome. Recent studies have yet to convincingly demonstrate HGH to be ergogenic.[94,96] Adverse effects of injectable HGH must also be considered, and include insulin resistance, glucose intolerance, oedema, and decreased endogenous HGH secretion.[97] Because of growth hormone's role in the regulation of lipoprotein (a), there are also cardiovascular concerns particularly with long-term use.[97]

IGF-1 is produced primarily by the liver and is thought to have a protein anabolic effect by enhancing amino acid uptake and accelerating transcription and translation.[98] IGF-1 is often mentioned together with HGH, as HGH stimulates IGF-1 gene expression in all tissues.[95] However, hepatic production of IGF-1 is regulated by factors other than HGH, such as nutritional status, and circulating IGF-1 levels should be considered more as a marker of HGH action on the liver than as the mechanism by which HGH exerts its effects.[95]

Research on actual IGF-1 in humans has been unconvincing of an anabolic effect. Yarasheski and colleagues found that even in individuals who doubled their circulating IGF-1 levels, there was no effect on the rate of protein synthesis, and no increase in strength.[99] Like HGH, however, OTC products that claim to contain IGF-1 are either precursors or alternate formulations that are unproven as performance enhancers. Adverse effects of these OTC products have not been studied, but elevated IGF-1 levels have been linked to lung cancer and colorectal cancer.[97,100] Injectable subcutaneous IGF-1 can cause temporomandibular joint discomfort, weight gain, dyspnoea and sinus tachycardia.[91]

Regarding HGH and IGF-1, neither agent can be classified as ergogenic unless further study is done on an athletic population, the necessity of which is questionable. The problem the clinician faces is marketing. What is marketed is not the actual researched drug, and patients are often unaware of this important fact. It is also unlikely that either agent would ever be legalised by a major sports organisation.

8. Proteins and Amino Acids

Arguably, proteins and amino acids are the most heavily marketed category of sports supplements. Despite the known role of amino acids and protein synthesis in the development of muscle hypertrophy and strength, the necessity of additional supplementation beyond diet is highly questionable. It is generally accepted that athletes have a greater daily protein requirement than sedentary people. The recommended daily allowance is 0.8 g/kg/day for adults, but ranges from 1.2–1.8 g/kg/day for athletes, with the higher range being reserved for strength athletes.[101-103]

Despite the acceptance of the additional protein needs of athletes, most athletes eat well enough to obtain this in their diet,[101,104] and there is little evidence to support additional consumption of protein or amino acids as performance enhancing. Williams et al.[105] studied seven untrained individuals supplemented with a glucose/amino acid product for 10 weeks. They utilised a clever design whereby alternate legs within the same individuals were trained on successive days, so each individual in a

training group served as his or her own control. This minimised the inter-individual variability inherent in other small sample size studies. The investigators found no strength benefits of the supplementation. In another study, Jentjens et al.[106] found that the addition of protein and amino acids to a carbohydrate diet did not enhance post-exercise muscle glycogen synthesis. A widely cited, double-blind, crossover study by Lemon et al.[104] in 1992 found that supplemental protein intake did not increase muscle mass or strength in novice bodybuilders.

The branched chain amino acids (BCAAs) are leucine, isoleucine, and valine. Theoretically, they decrease protein-induced degradation, which can lead to a greater fat-free mass.[102] The data on BCAAs as an ergogenic aid are not convincing. Davis et al. studied the effects of BCAA administration to a sports drink in individuals who performed intermittent, high-intensity running, with no beneficial effect noted.[107] Some studies have suggested that BCAAs may reduce exercise-induced muscle damage based on creatine phosphokinase and lactate dehydrogenase levels, but did not address performance.[108,109] Simply put, BCAAs, while an essential component of diet, are not ergogenic when taken in mass quantities as a dietary supplement. In fact, Wagenmakers makes an interesting argument that BCAAs may be ergo*lytic* (detrimental to performance) due to impedance of aerobic oxygenation.[110]

It is common for a new amino acid complex with a catchy brand name to be touted as the next magic bullet. The fact is that while proteins and amino acids are essential components of diet, studies on supplemental protein are not convincing. Patients wishing to take amino acid or protein supplements should be told that a proper diet is sufficient, and that dietary protein provides 20 essential and nonessential amino acids, including those that are marketed as 'ergogenic', such as leucine, isoleucine, lysine, alanine and glutamine.

9. Pyruvate

Pyruvate is a carboxylic acid produced by the metabolism of glucose. In 1990, Stanko et al. published two studies that involved upper and lower extremity endurance capacity in individuals who consumed pyruvate.[111,112] The results of these studies caught the eye of the supplement industry and pyruvate was aggressively marketed by supplement companies as being performance enhancing for endurance events. The proposed but unproven mechanism was that pyruvate enhances glucose oxidation.

Analysis of these studies, however, shows that both involved untrained individuals and very small sample sizes (8–10 individuals), and neither study used pyruvate alone. The upper extremity study[112] utilised dihydroxyacetone and pyruvate, while the lower extremity study[111] utilised dihydroxyacetone, pyruvate and a pre-event high carbohydrate diet. The dose of pyruvate utilised (25g) was also much higher than that marketed by supplement companies, which is usually 5g. High doses can cause gastric distress, which also brings into question the feasibility of such studies truly being double-blind. Furthermore, pyruvate is usually marketed alone. The pyruvate/dihydroxyacetone combination product is commonly known as DHAP.

Morrison and colleagues[113] did a recent study that incorporated a randomised double-blind crossover design (n = 7), and found that 7g of pyruvate for 7 days did not improve cycling performance time (approximately 90 minutes of cycling). Equally important, blood pyruvate levels did not even increase despite the supplementation. In a separate study by Morrison, published in the same paper,[113] nine recreationally active individuals ingested 7, 15, and 25g of pyruvate, but again no effect was found on blood pyruvate, glucose or lipid metabolism. This is of particular interest because pyruvate is also marketed as a weight-loss and cholesterol-lowering agent, neither of which has been proven.[114] Unlike the studies by Stanko et al., both of Morrison et al.'s studies involved trained individuals, which most sports clinicians find clinically more relevant.

Pyruvate cannot be classified as being ergogenic, and one review even called the marketing of pyruvate as 'economic fraud'.[114] The studies by Stanko et al.[111,112] may reveal some potential of high dose pyruvate/dihydroxyacetone in untrained individuals, but the doses necessary cast significant

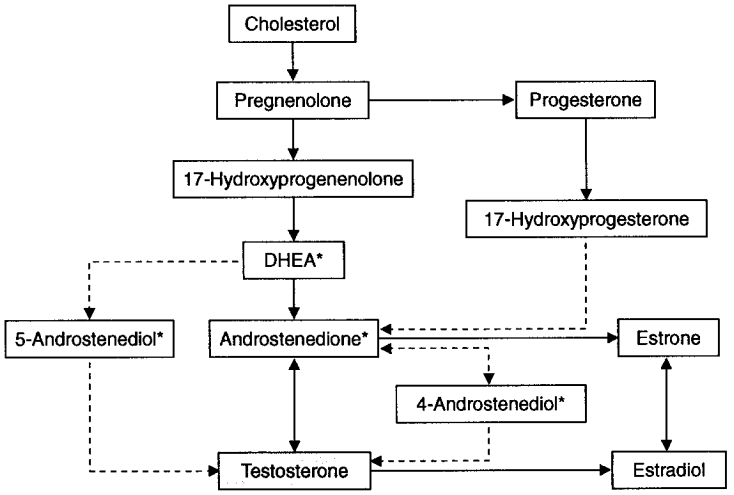

Fig. 1. The androgen and estrogen biosynthesis pathway. **DHEA** = dehydroepiandrosterone; * indicates androgenic precursors available over the counter.

doubt on the practicality of this regimen, least of all by trained athletes.

10. Androstenedione

If there were any doubts that society embraces its sports heroes, such doubts were quickly dispelled when sales of 'andro' skyrocketed after the revelation that a Major League Baseball player was using the supplement during a season where he broke the single season home run record. Research has shown, however, that this accomplishment cannot be attributed to the use of androstenedione.

It is common for an athlete to ask if androstenedione is a steroid. It is an 'androgenic' steroid, but it has not been proven to be an 'anabolic' (muscle building) steroid. In other words, it is not ergogenic. Specifically, androstenedione is an androgen/testosterone precursor (figure 1). Testosterone is synthesised from cholesterol via the δ-4 or δ-5 pathway, each of which involves androgenic precursors as intermediaries between cholesterol and the final testosterone product.[115,116] In the US, androgenic precursors are sold OTC, with androstenedione and dehydroepiandrosterone (DHEA) being the most popular.[115]

Oddly enough, androgenic precursors are not classified as drugs in the US and therefore fall under the loose guidelines of the Dietary Supplement Health and Education Act of 1994, meaning no FDA verification of product claims. There were no published studies on androstenedione prior to the 1998 McGwire story, but JAMA quickly published two well designed studies[117,118] that did not support an ergogenic effect, and only one of the two[117] demonstrated any increase in serum testosterone. Both studies, however, found significant increases in estradiol/estrogen synthesis, which is not surprising given estradiol's relationship to the biosynthesis of testosterone (figure 1).

In an extensive study, Broeder et al.[119] studied the effects of 200mg androstenedione daily for 12 weeks in men aged 35–65 years, who participated in a high-intensity resistance training programme. They found that serum testosterone levels increased at 1 month, but despite continued supplementation, returned to baseline levels by week 12, likely due to down-regulation of endogenous testosterone synthesis. More relevant is the fact that there was no change in body composition or strength compared with placebo. There were also adverse effects on high-density lipoprotein levels and coronary heart disease risk. Furthermore, estradiol levels increased by 97% in the treatment group due to a sustained increase in aromatisation leading to increases in estrogen synthesis.

Finally, a study by Rasmussen et al.[120] used isotopic tracer procedures to demonstrate that androstenedione does not promote muscle protein synthesis. In fact, under non-resistance training, Rasmussen found that the androstenedione group produced a greater degree of muscle protein breakdown than synthesis.

The lack of evidence supporting an ergogenic effect of androstenedione, combined with its adverse effects and legal ramifications, puts androstenedione on the not-recommended list of sports supplements. Additionally, androstenedione has been shown to cause a positive urine test for the anabolic steroid nandrolone.[121]

11. Dehydroepiandrosterone

DHEA is also an androgen precursor (figure 1). It is produced by the adrenal glands. As a precursor to androgenic steroids, DHEA may increase the production of testosterone and is marketed as having an anabolic steroid effect.

Brown et al.[122] examined the effects of DHEA on serum androgen levels and resistance training. Ten men (average age 23 years), were given a single 50mg dose of DHEA, and within 60 minutes had increased their androstenedione concentration by 150%. However, testosterone levels did not increase. Additionally, Brown et al. evaluated the effects of 150 mg/day of DHEA in 19 men (average age 23 years), while engaging in an 8-week resistance training programme. No changes in body composition or strength were found.[123]

While studies on adverse effects are lacking, there are reports of irreversible virilisation in women, including hair loss, hirsutism and voice deepening.[124] Men have reported irreversible gynecomastia, which may result from an elevation in estrogen levels. There is also concern that DHEA may increase the risk of uterine and prostate cancer due to prolonged unopposed estrogen and testosterone.[124]

Like androstenedione, DHEA is not classified as a drug, but rather a dietary supplement, and is therefore available OTC. Athletes should also be warned that any androgen precursor could alter the testosterone-epitestosterone ratio enough to exceed the 6 : 1 limit, which the IOC and NCAA enforce in their screening for exogenous testosterone use. It would be unfortunate for an athlete to be disqualified from competition for taking a product that does not have evidence to support an ergogenic effect to begin with.

12. Anabolic Steroids

Anabolic steroids began the athletic world's obsession with ergogenic aids. Anabolic steroids are simply the synthetic derivatives of the prototypical anabolic steroid, testosterone. They come in oral and injectable forms. Popular names include dianabol, nandrolone and stanozolol. The United States Congress classified anabolic steroids as a class III controlled substance in 1991. While it took many years for the medical community to admit it, the evidence is in: anabolic steroids work, but at a price.[125,126]

There have been several extensive reviews of anabolic steroids and their use in sport, and each has drawn similar conclusions regarding their anabolic effect as well as numerous adverse effects.[125-128] Briefly, anabolic steroids increase muscle mass by increasing muscle protein synthesis.[129] The exact mechanism is related to the increased nitrogen retention as a result of androgen excess. Via the metabolism of testosterone in the liver, an androgen receptor complex is formed which initiates the cellular transcription necessary for protein synthesis and muscle accretion.[125,128] There is also evidence to suggest that anabolic steroids increase erythropoiesis.[125]

Anabolic steroids are banned by the IOC, and the most utilised test is the ratio of testosterone to its metabolite, epitestosterone (T : E ratio), which is 1 in most men. The cut-off being 6 : 1 is generous and likely tempts athletes to test the limits.

Some recent studies have shed more light on anabolic steroids and their potential uses and abuses. Tamaki and colleagues demonstrated that anabolic steroids increase exercise tolerance.[130] While this was a rat model, it was the first study to address the relationship between protein synthesis and mitotic activity in skeletal muscle after anabolic steroid treatment, with and without exercise. There are also

potential medical uses of anabolic steroids, such as its use in genetic or HIV-induced muscle wasting disorders.[125,131]

Some concern has been voiced that elevated aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels should not be dismissed as always being steroid-induced in an anabolic steroid user. Dickerman et al. found that resistance training alone can lead to elevations in AST and ALT, but not glutamyl transpeptidase (GGT).[132] Clinicians should take note of this and include a GGT in the workup of any anabolic steroid user.

While there can be no denying the legitimate medical uses of anabolic steroids and the sometimes biased attitudes of physicians towards users, the ergogenic effect of anabolic steroids cannot justify their use in sport. Adverse health consequences are well established and include increased virilisation in women, menstrual irregularities, premature closure of growth plates, hirsutism, acne, aggressive behaviour, liver dysfunction and increased cardiovascular event risk.[125-128] A recent study found that only 17% of anabolic steroid users had normal spermatozoa.[133] There are also significant psychosocial aspects related to anabolic steroid use that cannot be overlooked. Kindlundh et al.[134] found significant associations between anabolic steroid use and immigrant status, low self-esteem, low school achievement, and use of prescription sedatives. On an encouraging note, Nilsson and colleagues[135] studied 16- to 17-year-old male anabolic steroid users, and found that discussion about appearance and attitudes helped to decrease steroid abuse. The take-home message is that clinicians should make every effort to counsel their patients.

13. Misleading Marketing

A discussion about sports supplements would not be complete without mentioning the dangers of misleading marketing. Green and colleagues evaluated 12 OTC anabolic-androgenic supplements using high pressure liquid chromatography.[136] Eleven of the 12 products (92%) had less product than what was labelled. Similar misleading labels (six of seven) were found in Catlin et al.'s study.[121] These findings are discouraging, but not surprising. It reflects the significant impact of the Dietary Supplement Health and Education Act of 1994. The passage of this Act, which allows any product not classified as a drug to bypass the FDA approval process, appears to have opened the door to unscrupulous business practices, and the studies by Green and Catlin suggest that a close re-evaluation of the Act is warranted. Most other countries, similar to the US, do not have strict regulation of supplements.

14. Conclusion

Many products are marketed as ergogenic despite a lack of evidence to support such claims. While a few have ergogenic potential, their applicability is limited to certain types of exercise and individual variability is a significant factor.

Antioxidants, proteins and amino acids are essential components of diet; however, additional oral supplementation does not increase endurance or strength. Caffeine is ergogenic in certain aerobic activities and is relatively safe. Laboratory studies on creatine show promise in repetitive anaerobic cycling sprints and maximal voluntary muscle contraction, but the research on running and swimming is not convincing of an ergogenic effect. Ephedrine and pseudoephedrine may have ergogenic potential but with a very high risk of adverse cardiovascular effects, and are appropriately banned by most sport governing bodies. EPO increases $\dot{V}O_{2max}$ and time to fatigue, but increases the risk of thromboembolic events. HMB may increase strength but more data are needed on trained individuals. HGH and IGF-1 decrease body fat and may increase lean muscle mass when given subcutaneously. Pyruvate is not ergogenic. The androgenic precursors androstenedione and DHEA have not been shown to increase any parameters of strength and have potentially significant adverse effects. Anabolic steroids increase protein synthesis and muscle mass but with many adverse effects, some irreversible. Labels describing content of supplements can be inaccurate and misleading. It must be emphasised that the population being 'treated' is not sick or diseased in any way, so it is important for the sports clinician to stay abreast

of the information in order to better advise their active patient population.

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Effect of sodium nitrate loading on electrolyte transport by the renal tubule

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KAHN, THOMAS, JUAN BOSCH, MARVIN F. LEVITT, AND MARVIN H. GOLDSTEIN. *Effect of sodium nitrate loading on electrolyte transport by the renal tubule.* Am. J. Physiol. 229(3): 746–753. 1975.— Effects of sodium nitrate were compared with sodium chloride loading on transport of electrolytes by the nephron. Maximal levels of free water clearance/glomerular filtration rate (C_{H_2O}/GFR) averaged 8.4% with nitrate loading and 14.4% with saline loading. Since ethacrynic acid and chlorothiazide exert their major natriuretic effect in the distal nephron, the increment in Na and Cl excretion produced by these agents was utilized as an index of Na and Cl reabsorbed beyond the proximal tubule. The administration of these agents resulted in an increase in fractional sodium excretion (C_{Na}/GFR) of 21.1%, urinary sodium excretion ($U_{Na}V$) of 1,126 μeq/min, and urinary chloride excretion ($U_{Cl}V$) of 848 μeq/min during nitrate loading compared with an increase in C_{Na}/GFR of 37.6%, $U_{Na}V$ of 2,362 μeq/min, and $U_{Cl}V$ of 2,397 μeq/min during saline loading. The smaller diuretic-induced increment in Na and Cl excretion in the nitrate studies suggests, as do the hydrated studies, that less Cl and Na are reabsorbed in the distal nephron during nitrate than saline loading. At every level of $U_{Na}V$, fractional bicarbonate reabsorption was higher, urine pH was lower, and urinary potassium excretion (U_KV) was higher in the nitrate studies. Thus, compared with saline loading, sodium nitrate decreases chloride and sodium reabsorption in the distal nephron. The higher hydrogen and potassium secretion in the nitrate studies may be consequent to the decreased ability of the distal nephron to reabsorb chloride.

ethacrynic acid; chlorothiazide; urine pH; saline loading; potassium transport; hydrogen transport; chloride reabsorption

CHRONIC ADMINISTRATION OF SODIUM NITRATE results in a metabolic alkalosis and a low plasma chloride (1, 17, 19, 24). The maintenance of the elevated plasma bicarbonate in this situation has been attributed primarily to an enhanced sodium-for-hydrogen exchange provoked by the need to conserve sodium in association with decreased availability of chloride for reabsorption because of the low plasma chloride (17, 24). It is of interest that with chronic sodium nitrate loading chloride excretion persists although plasma chloride levels are decreased (1). In acute studies with sodium nitrate loading a chloride diuresis develops (16, 27) that is greater than that noted with the administration of other sodium salts with the exception of sodium chloride (27). These studies suggest that nitrate, a semipermeable anion (16), may have a specific effect on the reabsorption of chloride. An earlier study indicated that during stable nitrate-induced alkalosis the capacity to generate free water reabsorption ($T^c_{H_2O}$) is decreased (34). The present studies were performed in order to more clearly delineate the effect of sodium nitrate loading on the reabsorption of chloride and to try to relate these observations with the effect of nitrate on the tubular transport of hydrogen and potassium.

METHODS

I) Sodium nitrate infusion under hydrated conditions. In five mongrel dogs anesthesia was induced with pentobarbital and maintained with a mixture of alpha-chloralose and urethan. Both ureters were cannulated through a suprapubic incision and the femoral artery was cannulated to obtain arterial blood samples. A constant infusion of creatinine was administered at 1.0 ml/min. Respiration was maintained stable with an endotracheal tube attached to a Harvard respirator. A solution of 2.5% glucose in water was administered through a catheter in the jugular vein at a rate of 10–20 ml/min until urine osmolality was less than 100 mosmol/kg water and no glucosuria was present. The infusion of glucose was then discontinued and a solution of 0.8–1.0% sodium nitrate was administered at approximately 10–15 ml/min in excess of urine flow rate. Clearance periods were obtained until an increase in the rate of infusion did not result in an increase in urine flow. At the end of the experiment the animals were in marked positive balance, similar to that produced in previous loading studies (33).

II) Saline studies. Twelve dogs were anesthetized and prepared surgically as described in *I*; 0.8% sodium chloride was administered initially at 10–15 ml/min and subsequently was increased progressively at a rate to exceed urine flow rate by 15–20 ml/min. Arterial blood was obtained in heparinized syringes and analyzed immediately for pH and P_{CO_2}. The P_{CO_2} in the blood was maintained stable within a range of 33–43 mmHg. Urine was analyzed immediately for pH and P_{CO_2}. Standard 10-min clearance periods were obtained.

In 6 of these 12 studies urine flow rate was allowed to remain stable for a period of 60 min or longer while the infusion was maintained constant at a rate of 15–20 ml/min in excess of urine flow rate. After two or three clearance periods during this stable state in three studies, 250 mg of chlorothiazide were administered intravenously and two 10-min clearance periods were obtained. Thereupon 50

mg of ethacrynic acid plus an additional 125 mg of chlorothiazide were administered intravenously and four consecutive 10-min clearance periods were obtained. In three studies 50 mg of ethacrynic acid plus 250 mg of chlorothiazide were administered simultaneously and four consecutive 10-min clearance periods were obtained.

In four additional studies 0.6% NaCl was infused until a stable urine volume was obtained and then 50 mg of ethacrynic acid were administered intravenously and four 10-min clearance periods were obtained.

III) Sodium nitrate studies. Fourteen dogs were anesthetized and prepared surgically as described in *I*; 1.0–1.1% sodium nitrate was infused at progressively increasing rates and clearance periods were obtained in a manner similar to that described under the saline studies in *II*.

As in the saline studies, when urine flow rates had stabilized for a period in excess of 60 min, 250 mg of chlorothiazide were administered intravenously in three studies and subsequently 125 mg of chlorothiazide plus 50 mg of ethacrynic acid were administered and four 10-min clearance periods were obtained. In three studies 50 mg of ethacrynic acid plus 250 mg chlorothiazide were administered intravenously in one bolus and four 10-min clearance periods were obtained.

In four nitrate loading studies, after the attainment of a stable urine flow rate, 50 mg of ethacrynic acid were administered intravenously and four 10-min clearance periods were obtained.

In three additional studies 0.8% NaCl was administered until a stable urine flow rate was achieved. Thereupon, the NaCl was discontinued and replaced by 1.0% $NaNO_3$ infused at the same rate. After an average of 2,240 ml $NaNO_3$ had been infused two periods with a stable urine flow rate were obtained. Subsequently 50 mg of ethacrynic acid were administered intravenously and four clearance periods were obtained.

Plasma and urine sodium and potassium were determined by Instrumentation Laboratories spectrophotometer and chloride by the Cotlove technique with the Buchler chloridometer. Creatinine was determined as outlined by Smith (32). Plasma bicarbonate was calculated by the Henderson-Hasselbalch equation with a pK' of 6.10. Urine bicarbonate was calculated with the use of urine P_{CO_2} and pH and with pK' determined according to the formula $pK' = 6.33 - 0.5\sqrt{(Na) + (K)}$ (18). Plasma and urine CO_2 content in some studies was also determined with the Natelson microgasometer. Titratable acid and ammonium in the urine were determined by the method of Jorgensen (21) with the Radiometer automatic titrator.

RESULTS

I) Sodium nitrate infusion: hydrated conditions. Minimal urine osmolality averaged 76 mosmol/kg water during water hydration. The GFR remained stable in these studies and averaged 42 ml/min in a single kidney. All results presented are from a single kidney. During the infusion of sodium nitrate, urine flow rate increased and free water clearance (C_{H_2O}) increased (Table 1). During this portion of the study, sodium excretion increased modestly but there was generally little increase in chloride excretion (Fig. 1, Table 1).

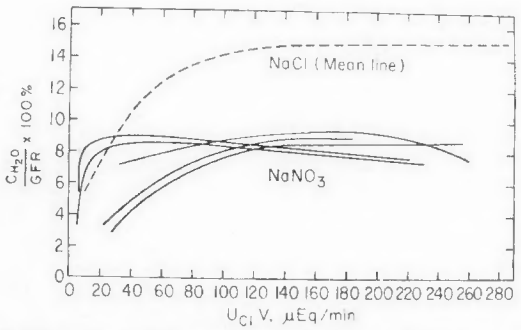

FIG. 1. Clearance of free water (C_{H_2O}) in milliliters per minute over glomerular filtration rate in milliliters per minute is plotted as a percent (C_{H_2O}/GFR × 100) versus chloride excretion in microequivalents per minute ($U_{Cl}V$). Mean line for saline studies was obtained from previously published studies (33).

Eventually, C_{H_2O} and C_{H_2O}/GFR reached maximal levels averaging 3.5 ml and 8.6%, respectively, while chloride excretion and sodium excretion increased progressively, suggesting that, in spite of increments in reabsorbable supply to the distal nephron, no further increase in distal transport occurred (Fig. 1, Table 1). In the presence of a poorly reabsorbable anion, the more conventional parameters of distal supply (C_{H_2O}/GFR + C_{Na}/GFR) or V/GFR are not a meaningful index of reabsorbable sodium reaching the distal nephron (22). The term (C_{H_2O} + C_{Cl})/GFR cannot be utilized as an index of the supply of sodium chloride reaching the distal nephron in the present studies because the plasma chloride was so different in the saline and nitrate studies that C_{Cl} is not comparable in the two groups of studies. Therefore, C_{H_2O}/GFR was plotted against $U_{Cl}V$, which is the chloride escaping reabsorption in the distal nephron. The excretion of unmeasured anion, presumably nitrate, increased as sodium excretion rose. Plasma chloride fell progressively in these studies from an average of 103 meq/liter during water hydration to an average of 68 meq/liter at the end of the study. Fractional chloride excretion averaged 9.7% at the end of the study. Plasma sodium remained in the range of 135–148 meq/liter. A representative study is given in Table 1.

The results from the infusion of sodium nitrate under hydrated conditions have been compared with the results obtained in previously published studies performed with hypotonic saline loading (33). The mean line for C_{H_2O}/GFR is shown from eight studies with 0.45% sodium chloride loading in which GFR averaged 39 ml/min and minimal urine osmolality averaged 67 mosmol/kg water. In the saline studies plasma sodium ranged from 127 to 150 meq/liter and plasma chloride averaged 113 meq/liter when chloride excretion averaged 200 μeq/min. Maximal C_{H_2O}/GFR averaged 14.4% in the saline studies (Fig. 1).

II) Saline studies. In the saline studies, GFR remained stable throughout each study, averaging 50 ml/min in the 12 studies. Plasma sodium concentration averaged 151 meq/liter in the control state and 152 meq/liter at the end of the study. Plasma potassium averaged 3.6 meq/liter initially and 3.2 meq/liter at the end of the study; plasma chloride tended to rise from an average of 111 meq/liter before the infusion to 128 meq/liter at the end of the infusion (prior to administration of diuretics). Average values for plasma bicarbonate generally decreased from 21.3 meq/

liter before the infusion to 15.2 meq/liter at the end of the infusion period. Sodium excretion was approximately equal to chloride excretion (Table 2). Bicarbonate excretion generally increased as sodium excretion progressively rose so that the fraction of the filtered load of bicarbonate that was reabsorbed declined (Fig. 2, Table 2). Urine pH initially declined with increasing sodium excretion, but subsequently stabilized with the average values for urine pH remaining consistently above pH 7.2 (Fig. 2). Titratable acid and ammonium excretion were low in the initial periods and then became negligible in both the saline and nitrate studies at flow rates above 3.0 ml/min per kidney. Therefore, these results were not analyzed further. Potassium excretion increased in association with the increase in sodium excretion (Fig. 3, Table 2).

In each of the six studies in which ethacrynic acid and chlorothiazide (distal blockade) were administered, the results of two or three periods obtained prior to the administration of these drugs were averaged and utilized as the control values for determining the effect of the diu-

TABLE 1. *Representative study with $NaNO_3$ loading under hydrated conditions*

Time, min	V, ml/min	GFR, ml/min	P_{osm}, mosM	U_{osm}, mosM	C_{H_2O}, ml/min	C_{H_2O}/GFR ×100%	P_{Na}, meq/liter	U_{Na}, meq/liter	$U_{Na}V$, μeq/min	C_{Na}/GFR ×100%	P_{Cl}, meq/liter	U_{Cl}, meq/liter	$U_{Cl}V$, μeq/min	C_{Cl}/GFR ×100%
−15			307				166				118			
−12	*Infusion of creatinine started and maintained at 1 ml/min*													
0	*2.5% Dextrose in water infused at 14 ml/min*													
145–155	3.8	55	273	61	2.9	5.3	146	6	22	0.3	99	3	12	0.2
158	*Discontinue dextrose in water; 0.9% $NaNO_3$ infused at 14 ml/min and rate increased progressively*													
165–175	4.9	52	271	56	3.9	7.5	144	9	44	0.6	98	1	5	0.1
187–197	5.7	48	271	63	4.4	9.2	145	9	51	0.7	96	1	6	0.1
207–217	6.1	50	269	73	4.4	8.4	145	19	116	1.6	97	2	12	0.2
230–240	6.7	51	272	95	4.4	8.6	149	30	201	2.7	94	4	27	0.6
254–264	8.6	55	273	118	4.9	8.9	149	41	352	4.3	91	9	77	1.5
268–278	8.4	53	273	137	4.2	7.9	148	50	420	5.4	90	12	101	2.1
278–288	9.5	54	274	145	4.5	8.3	147	59	560	7.1	88	13	124	2.6
298–308	10.5	54	274	155	4.5	8.3	147	71	745	9.5	88	16	168	3.5
358–368	11.0	53	274	169	4.2	7.9	146	78	859	11.1	78	16	176	4.3
387–397	12.8	55	275	188	4.0	7.3	144	86	1,100	13.9	73	18	230	5.7

V = urine flow; GFR = glomerular filtration rate = creatinine clearance; P_{osm}, U_{osm} = plasma and urine osmolality, respectively; C_{H_2O} = clearance of free water; P_{Na}, U_{Na} = plasma and urine sodium concentration; P_{Cl}, U_{Cl} = plasma and urine chloride concentration.

TABLE 2. *Representative study with 0.9% NaCl loading plus blockade*

Time, min	V, ml/min	GFR, ml/min	P_{Na}, meq/liter	$U_{Na}V$, μeq/min	C_{Na}/GFR ×100%	P_{Cl}, meq/liter	$U_{Cl}V$, μeq/min	C_{Cl}/GFR ×100%	P_K, meq/liter	U_KV, μeq/min	C_K/GFR ×100%	P_{HCO_3}, meq/liter	Plasma P_{CO_2}, mmHg	Urine pH	Urine P_{CO_2}, mmHg	U_{HCO_3}, meq/liter	$U_{HCO_3}V$, μeq/min	$(1-U_{HCO_3}V/GFR \cdot P_{HCO_3})$ ×100%
−30			147			106			3.3			21.0	40					
−28	*Infusion of creatinine started and maintained at 1 ml/min*																	
0	*0.9% NaCl infused at 15 ml/min and rate increased progressively*																	
67–77	1.1	44	145	90	1.4	112	92	1.9	2.9	21	17	19.3	43	7.21	36	11.9	13.1	98.5
102–112	3.7	43	146	152	2.4	112	133	2.8	3.0	24	19	18.1	40	7.36	41	16.5	61.1	92.2
140	*0.9% NaCl rate of infusion increased to 32 ml/min and maintained at this rate*																	
142–152	5.4	40	150	232	3.8	120	216	4.5	3.0	24	20	17.5	39	7.15	40	10.0	54.0	92.3
153–163	6.5	43	148	338	5.3	119	293	5.7	3.1	36	27	16.3	40	7.10	42	9.9	64.4	90.8
175–185	7.6	43	149	517	8.1	123	532	10.1	3.2	53	39	16.1	41	7.05	44	9.5	72.2	89.6
220–230	7.8	45	148	554	8.3	127	585	10.2	3.3	55	37	16.0	41	7.02	44	8.8	68.6	90.5
232–242	7.9	44	150	561	8.5	126	577	10.4	3.3	56	39	16.0	40	7.05	43	9.3	73.5	90.0
250–252	*50 mg ethacrynic acid plus 250 mg chlorothiazide administered intravenously*																	
256–266	14.4	40	149	1,944	32.6	133	1,973	37.1	3.3	122	93	15.5	41	7.09	46	12.3	177.1	71.4
267–277	21.8	40	152	3,205	52.2	132	3,313	62.8	3.4	157	115	15.8	42	7.01	43	9.5	206.8	67.4
277–287	20.2	39	153	2,909	48.7	133	3,030	58.4	3.1	154	127	14.7	41	6.97	43	8.7	176.2	69.5
288–298	19.7	40	149	2,837	47.8	131	2,916	55.6	3.3	146	110	14.8	40	7.02	44	10.0	197.0	66.8

Abbreviations as in Table 1; P_K, U_K = plasma and urine potassium concentration; P_{HCO_3}, U_{HCO_3} = plasma and urine bicarbonate concentration; $(1 - U_{HCO_3}V/GFR \cdot P_{HCO_3}) \times 100\%$ = percent of filtered load of bicarbonate reabsorbed.

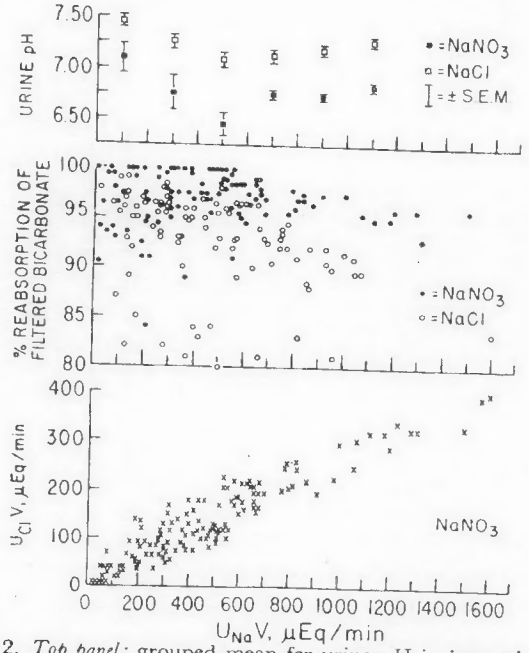

FIG. 2. *Top panel:* grouped mean for urine pH in intervals of 200 μeq/min of sodium excretion in studies performed with isotonic sodium nitrate and sodium chloride loading. *Middle panel:* fraction of filtered load of bicarbonate reabsorbed ($(1 - U_{HCO_3}V/GFR \cdot P_{HCO_3}) \times 100$) in individual periods plotted against sodium excretion in sodium nitrate and sodium chloride loading studies. *Bottom panel:* level of chloride excretion versus level of sodium excretion in sodium nitrate studies. In sodium chloride loading studies, level of chloride excretion was similar to that of sodium excretion in most periods (see Table 2).

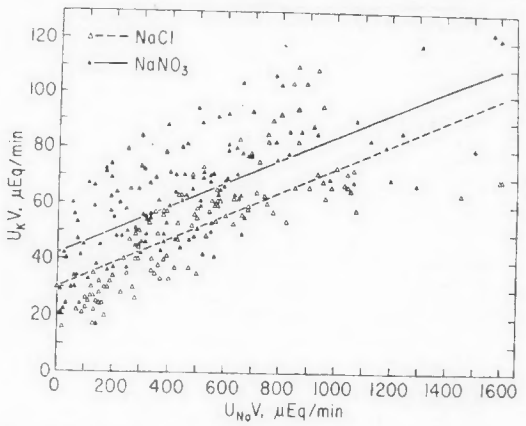

FIG. 3. Potassium excretion (U_KV) at every level of sodium excretion ($U_{Na}V$) is depicted in studies performed with isotonic sodium chloride and sodium nitrate loading. Equation for line representing saline points is $y = 0.043x + 30.2$ and for line representing nitrate points is $y = 0.044x + 42.0$. Intercepts are significantly different ($P < 0.001$).

retics. After the administration of ethacrynic acid plus chlorothiazide the three periods with the highest natriuresis were averaged and considered to be the results of control plus distal blockade. Results from the studies in which chlorothiazide alone was administered initially and then combined with ethacrynic acid were not different from those in which both diuretics were administered simultaneously, and therefore the results have been analyzed together. The average results of the control state and the postdiuretic state are given in Table 4. Fractional Na excretion increased from an average of 8.2% to an average of 45.8%, an increment of 37.6%. The increment in absolute sodium excretion averaged 2,362 μeq/min and the increment in chloride excretion averaged 2,397 μeq/min (Table 4). The increment in sodium and chloride excretion in the individual studies is shown in Fig. 4. It can be seen that the increment in chloride excretion was similar to the increment in sodium excretion in each study (Fig. 4).

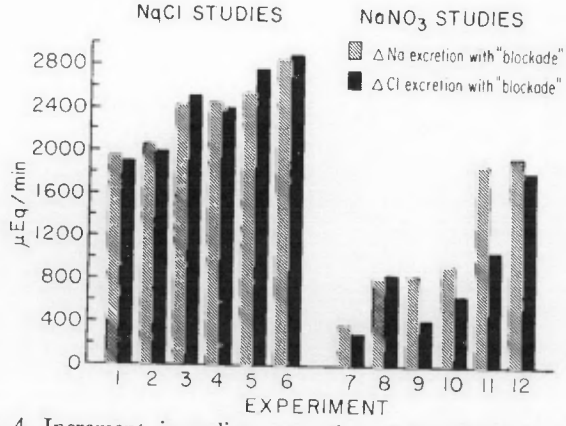

FIG. 4. Increment in sodium excretion and chloride excretion produced by administration of ethacrynic acid plus chlorothiazide during a stable diuresis is shown for 6 studies with sodium chloride loading (*1–6*) and 6 studies with sodium nitrate loading (*7–12*). Details of calculation used in determining increment in sodium and chloride excretion are given in text.

The studies in which 50 mg of ethacrynic acid were superimposed on a saline diuresis are summarized in Table 5, line 1. The increment in C_{Na}/GFR produced by ethacrynic acid averaged 32.7%.

III) Sodium nitrate studies. In the nitrate studies, GFR remained stable throughout the study, averaging 47 ml/min in the 14 studies. Plasma sodium concentration averaged 159 meq/liter prior to the infusion and 153 meq/liter at the end of the infusion; plasma potassium fell from an average control value of 3.8 meq/liter to 3.5 meq/liter at the end of the infusion. Plasma chloride concentration fell from an average level of 109 meq/liter prior to the nitrate infusion to an average of 79 meq/liter at the end of the infusion (prior to administration of diuretics). Plasma bicarbonate remained more stable than in the saline studies during the natriuresis, averaging 21.7 meq/liter prior to the nitrate infusion and 19.1 meq/liter at the end of the infusion. Despite the fall in plasma chloride, urine chloride excretion increased (Fig. 2, Table 3), although the bulk of the sodium was excreted with unmeasured anion, presumably nitrate. Urine bicarbonate excretion generally remained low throughout the nitrate diuresis, and consequently the fractional reabsorption of bicarbonate remained generally at levels of 95–100% (Fig. 2, Table 3). The fractional reabsorption of bicarbonate was higher in the nitrate studies than in the saline studies at every level of sodium excretion (Fig. 2). Urine pH at every level of sodium excretion was lower in the nitrate than in the saline studies (Fig. 2, $P < 0.05$ or less for each grouped mean). Potassium excretion also increased as urine sodium excretion increased and was higher than that noted in the saline studies at every level of urine sodium excretion (Fig. 3).

The six nitrate studies in which ethacrynic acid and chlorothiazide were administered were analyzed as out-

lined for the saline studies. The average results for the control and postdiuretic state are given in Table 4. After the administration of diuretics, the increment in C_{Na}/GFR averaged 21.1%, significantly less than the increment of 37.6% noted in the saline studies ($P < 0.005$). The increment in sodium excretion averaged 1,126 μeq/min, significantly less than the 2,362 μeq/min in the saline studies ($P < 0.001$), and the increment in chloride excretion averaged 848 μeq/min, significantly less than the increment of 2,397 μeq/min observed in the saline studies ($P < 0.001$). The increment in sodium and chloride in the individual studies is shown in Fig. 4. In five of the six studies, the increment in chloride excretion was less than the increment in sodium excretion (Fig. 4). The difference between the increment in sodium and chloride excretion probably reflects the increment in nitrate induced by the diuretics.

The results from the four studies in which 50 mg of ethacrynic acid were administered during a sodium nitrate diuresis are summarized in Table 5, line 2. The increment in $U_{Cl}V$, $U_{Na}V$, and C_{Na}/GFR produced by the ethacrynic acid averaged 776 μeq/min, 1,347 μeq/min, and 20.0%, respectively. The increments in $U_{Cl}V$ and C_{Na}/GFR are significantly ($P < 0.05$) lower in the nitrate than in the saline studies. The increment in $U_{Na}V$ is not statistically significantly lower ($P < 0.2$), probably consequent in part to an increase in GFR in the nitrate studies with a resultant increase in sodium nitrate excretion.

The results after the administration of 50 mg of ethacrynic acid from the three studies in which $NaNO_3$ replaced NaCl as the infusate are summarized in Table 5, line 3. In these studies the increment in $U_{Cl}V$, $U_{Na}V$, and C_{Na}/GFR averaged 810 μeq/min, 943 μeq/min, and 16.7%, respectively. The increment in $U_{Cl}V$, $U_{Na}V$, and C_{Na}/GFR in these studies is quite similar to the results obtained during the administration of only $NaNO_3$, whereas the increments $U_{Cl}V$ and C_{Na}/GFR are significantly ($P < 0.05$) lower than those obtained during saline alone. Control $U_{Cl}V$ averaged 637 μeq/min and control plasma chloride averaged 106 meq/liter in the NaCl-$NaNO_3$ studies (Table 5, line 3).

TABLE 3. *Representative study with 1% $NaNO_3$ loading plus blockade*

Time, min	V, ml/min	GFR, ml/min	P_{Na}, meq/liter	$U_{Na}V$, μeq/min	C_{Na}/GFR ×100%	P_{Cl}, meq/liter	$U_{Cl}V$, μeq/min	C_{Cl}/GFR ×100%	P_K, meq/liter	U_KV, μeq/min	C_K/GFR ×100%	P_{HCO_3}, meq/liter	Plasma P_{CO_2}, mmHg	Urine pH	Urine P_{CO_2}, mmHg	U_{HCO_3}, meq/liter	$U_{HCO_3}V$, μeq/min	$(1 - U_{HCO_3}V/GFR \cdot P_{HCO_3})$ ×100%
−35			148			107			3.3			20.5	42					
−33	*Infusion of creatinine started and maintained at 1 ml/min*																	
0	*1.0% $NaNO_3$ infused at 12 ml/min and rate increased progressively*																	
8–18	0.8	37	150	76	1.4	106	40	1.0	3.5	24	18	20.0	42	7.21	50	16.7	13.4	98.1
30–40	1.6	35	150	183	3.5	99	62	1.2	3.3	37	32	21.0	42	7.25	52	21.0	33.6	95.4
51–61	2.8	33	146	288	6.0	92	106	3.5	3.1	44	43	21.2	41	6.83	45	6.3	17.6	97.4
72–82	4.1	36	148	252	4.7	90	86	2.7	3.1	48	43	20.3	42	6.51	33	2.1	8.6	98.8
113–123	5.0	34	145	380	7.7	82	115	4.1	2.8	47	49	19.3	42	5.82	30	0	0	100
125	*1% $NaNO_3$ infusion increased to 28 ml/min and maintained at this rate*																	
135–145	6.2	38	148	505	9.0	82	124	4.0	2.8	49	46	19.0	44	6.06	36	0	0	100
184–194	6.3	35	149	539	10.3	77	120	4.5	2.7	52	55	18.1	40	6.20	31	1.1	6.9	98.9
194–204	6.4	35	149	560	10.7	76	122	4.6	2.7	52	55	18.0	41	6.02	32	0	0	100
208–210	*50 mg ethacrynic acid plus 250 chlorothiazide administered intravenously*																	
211–221	10.5	33	139	1,355	29.5	63	672	32.3	2.7	82	92	16.5	40	7.12	33	9.5	99.8	81.6
221–231	12.0	31	137	1,620	38.1	61	888	47.0	2.8	96	111	15.9	39	7.04	33	7.9	94.5	80.8
232–242	10.8	29	139	1,447	35.9	60	745	42.8	3.0	95	109	16.0	40	6.96	34	6.7	72.4	84.4
242–252	10.4	30	140	1,373	32.7	58	707	40.6	2.9	92	105	16.4	42	6.66	41	4.1	42.6	91.3

Abbreviations as in Tables 1 and 2.

TABLE 4. *Summary of blockade studies*

	GFR, ml/min	P_{Na}, meq/liter	$U_{Na}V$, μeq/min	C_{Na}/GFR ×100%	P_{Cl}, meq/liter	$U_{Cl}V$, μeq/min	C_{Cl}/GFR ×100%	P_K, meq/liter	U_KV, μeq/min	C_K/GFR ×100%	P_{HCO_3}, meq/liter
NaCl (6 studies)											
Control	41	150	472	8.2	126	502	9.9	3.6	54	34	14.4
EA + CTZ	42	149	2834	45.8	125	2899	54.7	3.4	146	102	13.5
$NaNO_3$ (6 studies)											
Control	43	141	646	10.2	73	180	4.2	3.6	67	44	16.6
EA + CTZ	42	138	1772	31.3	61	1028	38.7	3.6	107	75	15.2

Control = average of 2 or 3 periods during stable diuresis; EA + CTZ = average of 3 periods with maximal sodium diuresis obtained after administration of ethacrynic acid plus chlorothiazide.

TABLE 5. *Effects of ethacrynic acid*

	GFR, ml/min	P_{Na}, meq/liter	$U_{Na}V$, μeq/min	$C_{Na}/GFR \times 100\%$	P_{Cl}, meq/liter	$U_{Cl}V$, μeq/min
1) NaCl						
Control	42.9	134	666	11.8	112	682
50 mg EA	41.2	131	2,404	44.5	107	2,436
2) $NaNO_3$						
Control	40.4	149	1,013	17.0	64	248
50 mg EA	42.6	148	2,360	37.0	59	1,024
3) NaCl, then $NaNO_3$						
Control	50.3	155	1,155	15.0	106	637
50 mg EA	45.9	150	2,098	31.7	91	1,447

Control = average of 2 periods during diuresis; 50 mg EA = average of 2 periods with maximal sodium diuresis obtained after administration of ethacrynic acid; *group 1* refers to 4 studies with 0.6% NaCl loading; *group 2* refers to 4 studies with 1.0% $NaNO_3$ loading; *group 3* refers to 3 studies in which $NaNO_3$ replaced NaCl as infusate.

DISCUSSION

In the present studies, the effect of sodium nitrate loading on the reabsorption of sodium chloride in the nephron beyond the proximal tubule (distal nephron) was examined by two different techniques. In the first group of studies, the effects of sodium nitrate and sodium chloride loading on the generation of C_{H_2O} were compared. It is generally accepted that under hydrated conditions the generation of C_{H_2O} reflects an index of sodium chloride reabsorption within the distal nephron (7, 8, 15, 22, 29, 31, 33). Therefore, the finding that maximal levels of C_{H_2O}/GFR averaged 8.6% in the nitrate studies compared with an average of 14.4% in the saline studies suggests that the reabsorption of sodium chloride in the distal nephron is lower during sodium nitrate than sodium chloride loading (Fig. 1, Table 1) (33).

The finding of a lower C_{H_2O}/GFR in the nitrate studies may conceivably be consequent to a lower plasma chloride and therefore lower chloride supply to the distal nephron. Several lines of evidence are against this possibility. The increasing chloride excretion during sodium nitrate loading (Fig. 1, Table 1) suggests that distal chloride supply is progressively rising. Moreover in previous studies with sodium sulfate loading C_{H_2O}/GFR averaged 15% (22) and plasma chloride at the end of these studies averaged 77 meq/liter, a value comparable to that obtained in the nitrate studies. In other studies with mannitol (33) and sodium bicarbonate loading (29) C_{H_2O}/GFR attained levels of at least 15% despite reduced plasma chloride values. The failure of C_{H_2O} to rise therefore does not appear to be consequent to insufficient available permeable anion. It is also unlikely that the lower levels of C_{H_2O}/GFR in the nitrate studies can be attributed to high levels of intratubular nonreabsorbable solute. In the studies with sodium sulfate, sodium bicarbonate, and mannitol noted above (22, 29, 33), in which large quantities of solute excretion occurred, C_{H_2O}/GFR was similar to values attained with saline loading. Therefore, it does not appear that the lower C_{H_2O}/GFR in the nitrate studies is a consequence of a reduced chloride supply to the distal nephron or high levels of solute excretion. It is conceivable that nitrate in some way renders the collecting duct much more permeable to water even under hydrated conditions than does saline, but this seems unlikely, especially since C_{H_2O} did, in fact, rise initially with the administration of sodium nitrate (Fig. 1, Table 1). The finding of chloride in the urine associated with a lower C_{H_2O}/GFR in the nitrate studies therefore suggests that sodium nitrate loading interferes with sodium chloride reabsorption in the distal nephron.

In order to evaluate sodium reabsorption in the distal nephron by another technique, we utilized the combined effect of ethacrynic acid plus chlorothiazide to inhibit the reabsorption of sodium in the distal nephron. Earley, Martino, and Friedler (9) originally reviewed the rationale for the use of ethacrynic acid plus chlorothiazide as a "distal blockade" in order to study proximal tubule sodium transport, and several other authors have utilized this technique in a similar manner (13, 26). Numerous studies utilizing various techniques indicate that ethacrynic acid has its major natriuretic effect beyond the proximal tubule (4, 6, 7, 15, 23) and probably inhibits chloride and sodium reabsorption throughout the distal nephron (14, 23). Similarly, the natriuretic action of chlorothiazide appears to be predominantly in the distal nephron (6, 8, 14). Recent micropuncture studies in dog have suggested that ethacrynic acid may, under certain conditions, result in some inhibition in sodium reabsorption in the proximal tubule (5); other studies suggest that chlorothiazide may result in some inhibition of sodium reabsorption within the proximal nephron (10). Nonetheless, it is generally conceded by these authors and others that the major natriuresis produced by both ethacrynic acid and chlorothiazide is a result of the inhibition of sodium and/or chloride reabsorption beyond the proximal tubule (4–8, 10, 14, 15, 23, 31). It seems reasonable to assume, therefore, that the increment in sodium and chloride excretion produced by the administration of these drugs would reflect primarily an index of the reabsorption of sodium and chloride occurring in the distal nephron prior to the administration of the drugs.

In the saline studies the administration of ethacrynic acid plus chlorothiazide (distal blockade) resulted in an increment in C_{Na}/GFR of 37.6% and resulted in an increment in sodium excretion averaging 2,362 μeq/min and in chloride averaging 2,397 μeq/min (Table 4). In contrast, in the nitrate loading studies, with chloride present in the urine under control conditions, the administration of the diuretics resulted in an increment in C_{Na}/GFR of 21.1% and an increment in sodium and chloride excretion averaging 1,126 μeq/min and 848 μeq/min, respectively (Table 4). It is possible that these values for distal blockade are somewhat greater than the quantity of NaCl actually reabsorbed within the distal nephron since, in the presence of ethacrynic acid, some inhibition of proximal tubular reabsorption provoked by chlorothiazide may be reflected in the urine. However, the studies in which ethacrynic acid alone was administered demonstrated that the increment in C_{Na}/GFR was 32.7% during saline loading and 20% during nitrate loading (Table 5), implying that the major differences in NaCl reabsorption occurred at the ethacrynic acid site within the distal nephron. The smaller increment in fractional sodium excretion and absolute sodium and absolute chloride excretion provoked by ethacrynic acid

plus chlorothiazide in the nitrate compared with the saline studies therefore suggests that the reabsorption of sodium chloride in the distal nephron is less during sodium nitrate than sodium chloride loading.[1]

Both the free-water studies and the blockade studies suggest that the prompt progressive increase of chloride in the urine during a sodium nitrate diuresis (Fig. 2, Table 3) is probably consequent to a diminished reabsorption of chloride with sodium in the distal nephron. It is likely that overall distal chloride supply is lower in the nitrate than saline studies because of the lower plasma chloride during nitrate loading. The finding of a major chloruresis in the hydrated nitrate studies despite a lower distal chloride supply than with saline loading (Fig. 1, Table 1) and the chloruresis in the distal blockade studies prior to the administration of the diuretics (Fig. 2, Tables 3 and 4) imply that chloride escapes distal reabsorption in the presence of nitrate even with a lower distal chloride supply. Indeed, when plasma chloride averaged 106 meq/liter and $U_{Cl}V$ averaged 637 μeq/min in the studies in which sodium nitrate replaced sodium chloride as the infusate, a lower sodium chloride diuresis was provoked by ethacrynic acid than in the presence of a saline alone (Table 5, line 3). The decreased avidity for chloride in the distal nephron in the presence of nitrate may help to explain the previous observation of a greater chloruresis with sodium nitrate loading than with other nonchloride sodium salts (27).

It is generally believed that a major fraction of sodium transport beyond the proximal tubule takes place within the thick ascending limb, and recently it has been suggested that sodium reabsorption at this site is passive and dependent on the active reabsorption of chloride (3, 28). Moreover, considerable evidence suggests that the major site of action of the most potent diuretic agents and the predominant locus for the generation of C_{H_2O} is the ascending limb (14, 31). The experiments presented here therefore imply that sodium nitrate infusions decrease active chloride transport in the ascending limb and, possibly, throughout the distal nephron. The mechanism whereby nitrate alters the reabsorption of chloride at these sites is not clear.

The effects of sodium nitrate loading were also compared with the effects of sodium chloride loading on the transport of hydrogen and potassium. Saline loading resulted in an inhibition in the fraction of the filtered load of bicarbonate that was reabsorbed (Fig. 2), as had been reported by others (11). During sodium nitrate loading, however, fractional bicarbonate reabsorption was only slightly decreased (Fig. 2), implying that greater quantities of hydrogen are secreted during sodium nitrate than during sodium chloride loading. The persistently lower urine pH at every level of sodium excretion in the nitrate compared with the saline loading studies (Fig. 2) is in accord with this view. Potassium excretion at every level of sodium excretion was also higher in the nitrate than in the saline studies (Fig. 3).

The higher hydrogen and potassium secretion in the nitrate studies does not appear to be consequent to the unavailability of a permeant anion in the distal nephron since considerable quantities of chloride were excreted throughout the nitrate studies (Fig. 2). It is possible, however, that the higher hydrogen and potassium secretion is consequent to the decreased reabsorption of chloride with sodium during the nitrate loading studies throughout the distal nephron. During volume expansion with sodium chloride, sodium reabsorption is increased in the distal nephron primarily as sodium chloride (20, 25, 31, 33). During volume expansion with sodium nitrate, however, the ability of the distal nephron to increase chloride reabsorption appears to be diminished. Sodium may be reabsorbed either in association with an anion or in exchange for hydrogen and potassium (12). If the ability to reabsorb chloride is impaired during nitrate loading, an increase in the exchange of sodium for hydrogen and potassium may permit the distal nephron to increase sodium reabsorption.

The present observations may also help to explain the development of an alkalosis with a low plasma chloride during chronic sodium nitrate administration (1, 17, 19, 24). The continued excretion of chloride in these studies (1), despite the low plasma chloride, may partly be caused by a nitrate-induced decrease in transport of chloride and sodium within the distal nephron. A diminished reabsorption of chloride in the distal nephron may provoke an acceleration of sodium for hydrogen and potassium exchange and thus result in the development and maintenance of an increased plasma bicarbonate. It has been suggested that a diminished chloride supply to the distal nephron may result in an accelerated sodium-for-hydrogen exchange in a condition of marked avidity for sodium reabsorption (30). The present studies suggest that a decreased capacity of the distal nephron to reabsorb chloride, despite adequate chloride supply, may produce results similar to that noted with diminished chloride supply. An increase in hydrogen and potassium secretion may develop even in the absence of volume depletion and may reflect a persistent stimulus for sodium reabsorption in the distal nephron regardless of the extracellular volume.

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[1] Preliminary studies utilizing the technique of distal blockade during sodium sulfate and sodium bicarbonate loading have suggested that distal sodium chloride reabsorption is similar to that noted with saline loading (2).

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The present observations may also help to explain the development of an alkalosis with a low plasma chloride during chronic sodium nitrate administration (1, 17, 19, 24). The continued excretion of chloride in these studies (1), despite the low plasma chloride, may partly be caused by a nitrate-induced decrease in transport of chloride and sodium within the distal nephron. A diminished reabsorption of chloride in the distal nephron may provoke an acceleration of sodium for hydrogen and potassium exchange and thus result in the development and maintenance of an increased plasma bicarbonate. It has been suggested that a diminished chloride supply to the distal nephron may result in an accelerated sodium-for-hydrogen exchange in a condition of marked avidity for sodium reabsorption (30). The present studies suggest that a decreased capacity of the distal nephron to reabsorb chloride, despite adequate chloride supply, may produce results similar to that noted with diminished chloride supply. An increase in hydrogen and potassium secretion may develop even in the absence of volume depletion and may reflect a persistent stimulus for sodium reabsorption in the distal nephron regardless of the extracellular volume.

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STATE-SPONSORED RESEARCH ON CREATINE SUPPLEMENTS AND BLOOD DOPING IN ELITE SOVIET SPORT

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ABSTRACT The former Soviet Union began participating in international sport after World War II and soon achieved a dominant position in the Olympic Games and other competitions. The success of Soviet athletic programs led to charges of unfair practices but, because of secrecy surrounding Soviet research in exercise biochemistry, it has been difficult to substantiate these charges. This article presents previously restricted information regarding the development and use of creatine supplements and blood doping in the USSR. Early work by Olexander Palladin established the role of creatine in muscle function. In the 1970s, Soviet scientists showed that oral creatine supplements improved athletic performance in short, intense activities such as sprints. Subsequent studies in the West substantiated these investigations and have led to the widespread acceptance and use of creatine supplements to enhance muscle function and athletic performance. In addition, however, the Soviet government supported the development of blood doping, which is banned by the International Olympic Committee. Blood doping was pervasive in the USSR in the 1970s and 1980s, and was used by many Soviet athletes in the 1976 and 1980 Olympic Games. Open publication and discussion may help to prevent the abuses that can come from secret scientific research.

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THE COUNTRIES OF THE FORMER SOVIET UNION have a long history of research in exercise biochemistry but, because of the inaccessibility of the journals, lack of familiarity with the languages, and the secrecy surrounding this work, this research is not well known in the West (Kalinski and Dunbar 2000). Communication between Soviet and Western scientists before World War II was minimal, being restricted to a few conferences in the USSR. Later, during the Cold War, sharing of research between the two superpowers was not encouraged because of the intense rivalry between the United States and the USSR in the Olympic Games. This article presents information that was previously restricted and inaccessible in the West concerning research in sport biochemistry and the development of performance-enhancing nutritional supplements and procedures in the former USSR.

From the 1950s onward, dedication to Soviet athletic success was accompanied by the development of research in exercise biochemistry and physiology. These research fields became integral parts of the athletic agenda after World War II, when sport institutions received specific research assignments from the State Sport Committee of the USSR, an arm of the Soviet government. By the 1970s, there were 28 Institutes of Physical Education in the USSR. Despite having to operate within a totalitarian system, some of these programs became extremely successful. In general, sport institutions were directed to develop scientific sport programs for Olympic athletes rather than basic health and fitness programs for the general population. Some studies, such as the development of biochemical methods to monitor the training of elite athletes, and evaluation of the effects of nutritional ergogenic aids on metabolism and sport performance, were not restricted and were published in the open literature (Kalinski and Rogozkin 1988). Other aspects of Soviet sport research, however, including research on blood doping and its use by Soviet Olympic competitors during the Olympic Games in the 1970s and 1980s, were carried out in secret and are still not well known in the West.

CREATINE

Creatine was discovered in 1832, as a constituent of muscle. Early research on creatine was limited; it took almost a century before phosphocreatine and creatine kinase, the enzyme that catalyzes the synthesis and breakdown of phosphocreatine, were discovered. The pioneering studies of the role of creatine in skeletal muscle function were undertaken by Olexander Palladin. Palladin was educated at St. Petersburg University, where he studied under the supervision of Ivan Pavlov. After earning his bachelor's degree, Palladin moved to the Eastern Ukraine, where he was appointed professor at Kharkov University. Palladin became a leading muscle biochemist. In 1916, he published a monograph *Biosynthesis and excretion of creatine in animals*, and in 1929 he founded the Ukrainian Biochemical Research Institute, where he served as director until his death in 1972. Palladin had a

distinguished career in the Ukraine: he was elected academician and was appointed president of the Ukrainian Academy of Sciences after the Institute was moved to Kiev, the capital of Ukraine, in 1931. Several scientists from this Institute have moved to the United States, where they have reestablished successful research careers.

In now classic studies, Palladin and his associates showed that the concentrations of creatine and phosphocreatine in muscle vary with muscle contraction strength and the degree of training, and that chronic exercise leads to increases in both creatine and phosphocreatine (Lachno 1938; Palladin 1935, 1937; Palladin and Epelbaum 1928; Palladin and Ochrimenko 1938; Palladin and Sihalova 1934). In addition, they observed that fast-twitch, white muscle fibers contain more phosphocreatine than slow-twitch, red fibers. Thus, they established that the phosphocreatine content depends on the functional state of the muscle fibers (Palladin and Ferdman 1927). Palladin (1937) predicted that understanding the biochemistry of exercising muscle would lead to a means for enhancing exercise performance. This prediction was realized most strikingly and specifically with regard to the roles of creatine and phosphocreatine in muscle function.

During the 1970s and 1980s, the State Sport Committee directed several elite research institutions to conduct a broad range of investigations aimed at determining the effects of ergogenic dietary supplements and their possible use by Soviet athletes who were preparing to compete in the Olympic Games and other international competitions. The Central Institute of Physical Culture in Moscow initiated a long-term research program to characterize the role of creatine in muscular performance and its use to enhance muscle function, and Soviet scientists redirected their research from academic studies on creatine metabolism in animals to applied studies on the effects of creatine supplements on human physical performance (Volkov 1990).

In initial studies, creatine supplements were given to runners, especially those competing in 100- and 200-meter sprints. Creatine (125 mg/kg body weight per day) was shown to enhance biochemical and physiological measures of both aerobic and anaerobic metabolism as well as exercise performance in these athletes. Members of the USSR national track and field team who took creatine supplements improved their performance in the 100-meter dash by 1 percent and in the 200-meter sprint by 1.7 percent (Volkov 1990, 1991). Of course, an increase of a fraction of a second in competition can make the difference in setting a world record or winning "the gold." As a result of these studies, the Central Institute of Physical Culture officially recommended the use of creatine supplements to enhance physical capacity and the efficacy of exercise training, and USSR national athletes were routinely given these supplements (Volkov 1990).

These studies in the Soviet Union preceded similar investigations in the West, where scientists used muscle biopsy and biochemical methods to determine the effects of creatine on muscle energy metabolism. The interest in creatine supple-

ments in the West stems from the study of Harris, Soderlung, and Hultman (1992), who showed that oral creatine is absorbed and increases the concentrations of creatine and phosphocreatine in muscle in normal subjects. Subsequent studies confirmed that creatine supplementation improved exercise performance involving short periods of intense activity (Demant and Rhodes 1999; Roundtable 2000; Williams, Kreider, and Branch 1999). These studies led to the use of creatine supplements to enhance muscle function and to aid exercise performance. Creatine was used by Western athletes in the 1992 Olympic Games in Barcelona, and it has become one of the most widely used non-doping exercise-enhancing dietary supplements in sport (Anderson 1993; Demant and Rhodes 1999; Smith et al. 1999; Williams, Kreider, and Branch 1999). Worldwide, it has been estimated that athletes of all levels, from professional to recreational, consume more than 2.5 million kg of creatine per year (Roundtable 2000); sales revenues in the United States alone are estimated at as much as $100 million per year (Williams, Kreider, and Branch 1999).

BLOOD DOPING

Creatine appears to be beneficial for short, intense activity but not for endurance exercise—indeed, it may be disadvantageous for endurance type activities (Demant and Rhodes 1999). Because Soviet authorities were interested in success in all Olympic events, they sought out another exercise-enhancing strategy for endurance sports. Blood doping—the removal and storage of blood, and its reinfusion at a later date—is the strategy they pursued. Blood doping enhances the oxygen-carrying capacity of blood and enhances performance in endurance exercise (Robergs and Roberts 1997, p. 471). Although Soviet athletes had long been suspected of using blood doping, there was no proof, and Soviet authorities always denied using this procedure. And while several authors discussed blood doping in sport, none of these sources contained specific documentation about blood doping research in the USSR (Todd and Todd 2001; Voy 1990; Wilson and Derse 2001). Nonetheless, such research did exist. The research program involved the highest levels in sports institutions of the USSR, including the Central Institute of Physical Culture in Moscow and the Central Institute of Hematology and Transfusiology, and was carried out over a number of years. It also involved many of the country's elite athletes, including Olympic competitors, from different sports disciplines (Volkov 1990). The results of this clandestine government-sponsored research—including information pertaining to the use of blood doping during the 1976 Olympic Games—were restricted from open publication. Only 14 years after blood doping was used at the Olympic Games were the results of this research finally allowed to be made public—and then only in the form of an abbreviated Ph.D. dissertation report (Volkov 1990).

Studies described by Volkov (1990) were performed with two groups of athletes: middle- and long-distance runners who were members of the USSR

national team, and members of the national swim team of the Russian Republic (which was the largest republic in the USSR). Two blood-doping protocols were run. In one, 450 ml of whole blood was removed, stored for 18 to 22 days, and reinfused in the athletes. In the other, 450 ml of blood was withdrawn three times, and erythrocytes were separated, frozen, and stored. After 20 to 50 days, the red cells were thawed and reinfused into the athletes. Aerobic and anaerobic powers were assessed before and after blood letting, before reinfusion, and at different times after reinfusion. In one study, swimmers showed a dramatic increase in maximal oxygen consumption following blood doping (Volkov 1990). Maximal oxygen consumption is, of course, an important component of endurance exercise. In addition, for reasons that aren't clear, anaerobic capacity also increased. Swimmers showed their best improvement in performance, about 3 percent, two to three days after reinfusion of thawed erythrocytes; after that time, there was a small decrease in performance. Runners showed similar improvements in running times, but their performance was maximal eight to 10 days after reinfusion. Although the reasons for the different effects of blood doping on different types of athletic performance were not explained in the dissertation report, Volkov recommended that these differences be taken into consideration when using blood doping to prepare athletes for competition.

Volkov's doctoral dissertation report makes clear that the practice of blood doping was pervasive in the USSR in the 1970s and 1980s. Middle- and long-distance swimmers, cyclists, rowers, skiers, biathlon athletes, and skaters all used this procedure in the 1976 and 1980 Olympic Games. Blood doping was then—and is still—banned by the International Olympic Committee, but it is difficult to detect. The Committee has also banned erythropoietin, which induces the production of red blood cells. This was the drug of choice for long-distance speed skaters and skiers at the 2002 Winter Olympics in Salt Lake City; erythropoietin use caused the Russian cross-country skier Natalia Baranova-Masolkina to be removed from the Olympics.

ETHICAL CONSIDERATIONS

Creatine supplementation is apparently safe and is not banned by international sports committees. Blood doping, on the other hand, does carry some risk. One of the most serious risks is that of heart attack. The reinfusion of large amounts of erythrocytes may compromise blood flow, particularly through atherosclerotic coronary arteries. (Even elite athletes can develop atherosclerosis; recently, a young Russian figure skater, an Olympic medalist, died of a heart attack during a routine practice session.) An increased hematocrit might also impair blood flow to the brain and precipitate a stroke. For these reasons, blood doping is banned by the International Olympic Committee. The American College of Sports Medicine (1987) has called the use of blood doping unethical and unjustifiable (see also Sawka et al. 1996). In promoting blood doping, Soviet authorities were

clearly acting unethically, putting their athletes' lives at risk and violating the rules of Olympic competition.

Because doping is difficult to detect, Soviet officials, coaches, and sport scientists had a cynical attitude about the use of doping procedures. Portugalov, a leading Soviet-era sport scientist specializing in pharmacological research, made a typical statement describing the position of some corrupt Soviet officials, coaches, and scientists: "Basically, the question of a drug test's result has no meaning whatsoever . . . if a test is negative it only means that the pharmacological preparation was done correctly. If it is positive, then the coach is an idiot" (Kidd, Edelman, and Brownell 2001). On another occasion, Anatoliy Akimov, professor at the Moscow Institute of Physical Culture, made a sensational comment to a major Soviet newspaper, *Soviet Sport*. In his words, "an army of medical professionals behind closed doors" was working in the USSR on the development of doping in sports. When asked if this meant that the Soviet officials "consciously employed doping, instead of fighting doping," he replied, "Certainly. And in such manner doping was pervasive even in young teams" (Kalinski 1991).

Although Soviet research on blood doping has provided valuable insights into exercise performance, its clandestine use has tainted athletic victories. The revelations about the research and its results provide convincing reasons for encouraging international scientific discourse. Understanding the effects of exercise on muscle function and metabolism is valuable for maintaining human health, but the corruption of the processes that lead to such knowledge can undermine both science and the goal of fair play. As Baron Pierre de Coubertin said, the most important consideration about the Olympic Games is not winning, but taking part (Lucas 1992).

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Inorganic Nitrate Supplementation Lowers Blood Pressure in Humans

Role for Nitrite-Derived NO

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Abstract—Ingestion of dietary (inorganic) nitrate elevates circulating and tissue levels of nitrite via bioconversion in the entero-salivary circulation. In addition, nitrite is a potent vasodilator in humans, an effect thought to underlie the blood pressure–lowering effects of dietary nitrate (in the form of beetroot juice) ingestion. Whether inorganic nitrate underlies these effects and whether the effects of either naturally occurring dietary nitrate or inorganic nitrate supplementation are dose dependent remain uncertain. Using a randomized crossover study design, we show that nitrate supplementation (KNO_3 capsules: 4 versus 12 mmol [n=6] or 24 mmol of KNO_3 (1488 mg of nitrate) versus 24 mmol of KCl [n=20]) or vegetable intake (250 mL of beetroot juice [5.5 mmol nitrate] versus 250 mL of water [n=9]) causes dose-dependent elevation in plasma nitrite concentration and elevation of cGMP concentration with a consequent decrease in blood pressure in healthy volunteers. In addition, post hoc analysis demonstrates a sex difference in sensitivity to nitrate supplementation dependent on resting baseline blood pressure and plasma nitrite concentration, whereby blood pressure is decreased in male volunteers, with higher baseline blood pressure and lower plasma nitrite concentration but not in female volunteers. Our findings demonstrate dose-dependent decreases in blood pressure and vasoprotection after inorganic nitrate ingestion in the form of either supplementation or by dietary elevation. In addition, our post hoc analyses intimate sex differences in nitrate processing involving the entero-salivary circulation that are likely to be major contributing factors to the lower blood pressures and the vasoprotective phenotype of premenopausal women. (***Hypertension.*** **2010;56:274-281.)**

Key Words: clinical science ■ diet ■ NO ■ endothelium ■ blood pressure

Cardiovascular disease (CVD) is the biggest killer worldwide and is likely to increase in proportion as the non-Western world adopts a Western lifestyle (World Health Organization, fact sheet 317, www.who.int). Hypertension is a major risk factor for CVD and is predicted to reach a global prevalence of 30% by 2025.[1] Because blood pressure (BP) remains elevated in ≈50% of all treated hypertensive patients,[2,3] novel and cost-effective therapeutic strategies are urgently required for the treatment of this condition. In this regard, over the last decade, there has been a major initiative in the Western world to increase the public consumption of vegetables (Department of Health United Kingdom, 5 a day, www.nhs.uk/5aday) in part, as a strategy to prevent CVD.[4] This approach has been taken because epidemiological,[5] cohort,[6,7] and trial-based data[8,9] demonstrate that increased consumption of a vegetable-rich diet confers protection from CVD, including hypertension. However, the exact mechanisms of the BP-lowering and protective effects of such a diet remain uncertain.

Large-scale clinical trials have failed to show a beneficial cardiovascular effect of several different nutrients found in vegetables, including antioxidant vitamins and folate.[10,11] More recently, attention has been directed toward other possible elements in vegetables that may have a role, including inorganic nitrate.[12] In 2006, Larsen et al[13] demonstrated that supplementation of healthy volunteers with sodium nitrate resulted in a decrease in diastolic BP (DBP) but not systolic BP (SBP). More recently, we have shown that consumption of beetroot, which is a high nitrate-containing vegetable, also exerts a number of beneficial effects in healthy volunteers, including lowering of both SBP and DBP and protection of the endothelium from ischemia-reperfusion (IR)–induced endothelial damage.[14]

The activity of orally ingested inorganic nitrate is thought to lie in its conversion to nitrite by facultative bacteria found

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on the dorsal surface of the tongue.[15] The swallowing of this nitrite-rich saliva permits entry of nitrite into the circulation via the stomach, and then, once within the circulation, nitrite is thought to be converted to the potent vasodilator NO.[16–18] Evidence suggests that this circuit of bioactivation results in both vasodilator effects and protection against IR injury.[19] However, whether the beneficial cardiovascular effects of beetroot juice are specifically attributable to the dietary nitrate content of beetroot and whether the effects of either naturally occurring dietary nitrate or inorganic nitrate supplementation are dose-dependent remain uncertain.

Herein, we have investigated whether the effects of dietary provision of inorganic nitrate is recapitulated using potassium nitrate capsules and whether the effect of inorganic nitrate on circulating nitrite/nitrate levels, BP, and endothelial function is dose and NO dependent in healthy volunteers.

Methods

Volunteers

The studies were granted full ethical approval by the local research ethics committee. All of the subjects gave informed consent after satisfying the inclusion criteria (please see the online Data Supplement at http://hyper.ahajournals.org).

BP Studies

Volunteers were entered into 1 of 3 different studies. In the first study, 21 subjects were randomized to receive potassium nitrate capsules (KNO_3: 24 mmol giving 1488 mg of nitrate; Martindale Pharmaceuticals) and an equivalent dose of potassium chloride (KCl, Martindale Pharmaceuticals) with 500 mL of low nitrate-containing water (nitrate: 61.2±1.9 μmol/L; nitrite: 0.20±0.03 μmol/L; Zepbrook Ltd) in this double-blind, crossover study. In a separate study, 6 additional individuals were randomized to receive either 4 or 12 mmol (248 or 744 mg of nitrate, respectively) of KNO_3 with 500 mL of water in an open-label, crossover study. A further randomized, open-label, crossover study was performed in 9 healthy subjects to investigate dose dependency of the effects of beetroot juice–derived nitrate relative to our previous findings where 500 mL of juice were administered.[14] Volunteers received either 250 mL of beetroot juice (James White Drinks Ltd) or 250 mL of water. In all of the groups, blood samples were taken and BP determined at baseline and then at specific intervals for ≤24 hours.

Flow-Mediated Dilatation Study

The impact of an IR insult on endothelial function was assessed in 12 healthy subjects by measuring brachial artery diameter in the nondominant arm in response to reactive hyperemia (please see the online Data Supplement). Subjects were randomized in a double-blind (for capsules only) crossover study design to receive either 24 mmol of KNO_3 or KCl with 500 mL of water and, on another occasion, 250 mL of beetroot juice 90 minutes before ischemia.

BP Measurements

All of the BP and heart rate (HR) measurements were taken in triplicate in the seated position using an Omron 715IT before and after capsule, beetroot juice, or water ingestion for ≤24 hours (please see the online Data Supplement).

Blood Sampling

Blood samples were taken at baseline; then after capsule, beetroot juice, or water ingestion, every 30 minutes up to 3 hours; then in some studies hourly from 3 to 6 hours; and then again at 24 hours (please see the online Data Supplement).

Measurement of Plasma Nitrate/Nitrite and cGMP Concentration

Plasma nitrite and nitrate (NOx) concentration were measured using ozone chemiluminescence (please see the online Data Supplement). cGMP was determined using an enzyme immunoassay (cGMP EIA Biotrak System, GE Healthcare UK Ltd) according to the manufacturer's instructions.

Statistical Analysis

The data were analyzed by an individual who was blinded to the different interventions, using Graphpad Prism software version 5. All of the data are expressed as mean±SEM, unless otherwise specified. For BP measurements and plasma nitrate and nitrite concentration, repeated-measures ANOVA was used, with Dunnett posttest for comparison to baseline control and Bonferroni post test for comparison between groups at individual time points. Unpaired *t* tests were used for comparisons of baseline statistics. For flow-mediated dilatation and cGMP responses, repeated-measures ANOVA followed by Bonferroni posttests for individual group comparisons was used. Determinations of correlations between plasma nitrite or nitrate concentration with changes in SBP were completed using the Pearson correlation coefficient analysis.

Results

There were no significant differences in the general characteristics of the individuals recruited for the separate phases of the BP study (Table S1, available in the online Data Supplement). Beetroot juice was generally well tolerated by the subjects. The nitrate concentration in the beetroot juice was 22.4±3.8 mmol/L, whereas nitrite concentration was <50 nmol/L. Capsules were well tolerated in general, although one volunteer, who had not taken toast with the capsules, was treated for gastritis after consumption of capsules. This individual was unblinded and withdrawn from the study. On unblinding, it was discovered that gastritis occurred after taking chloride capsules. All of the subsequent subjects were made to take toast with the capsules, and there were no further adverse effects.

Dose-Dependent Increases in Circulating NO_3^- and NO_2^- After Oral Inorganic Nitrate Capsule Ingestion

After ingestion of KNO_3 capsules (24 mmol), there was a rapid (within 30 minutes) increase in circulating plasma nitrate concentration, peaking at 3 hours and remaining significantly elevated at 24 hours (Figure 1A). In contrast, the rise in plasma nitrite concentration was moderate, followed by a slower time course and significantly raised levels first evident at 1.5 hours, plateauing at ≈2.5 hours, sustained to 6 hours, and remaining elevated at 24 hours (Figure 1B). These effects of KNO_3 were dose dependent with plasma nitrate concentration elevated above baseline by ≈35-, 27-, and 7-fold after administration of 24, 12, and 4 mmol of KNO_3, respectively. The rises in plasma nitrite concentration also showed dose dependency, albeit with a more moderate rise of a 4.0-, 2.0-, and 1.3-fold increase, respectively (Figure 1).

Inorganic Nitrate Supplementation Elevates Plasma cGMP Concentration

Plasma cGMP concentration was significantly raised compared with baseline at 3 and 24 hours after ingestion of KNO_3 capsules (24 mmol; Figure 1E).

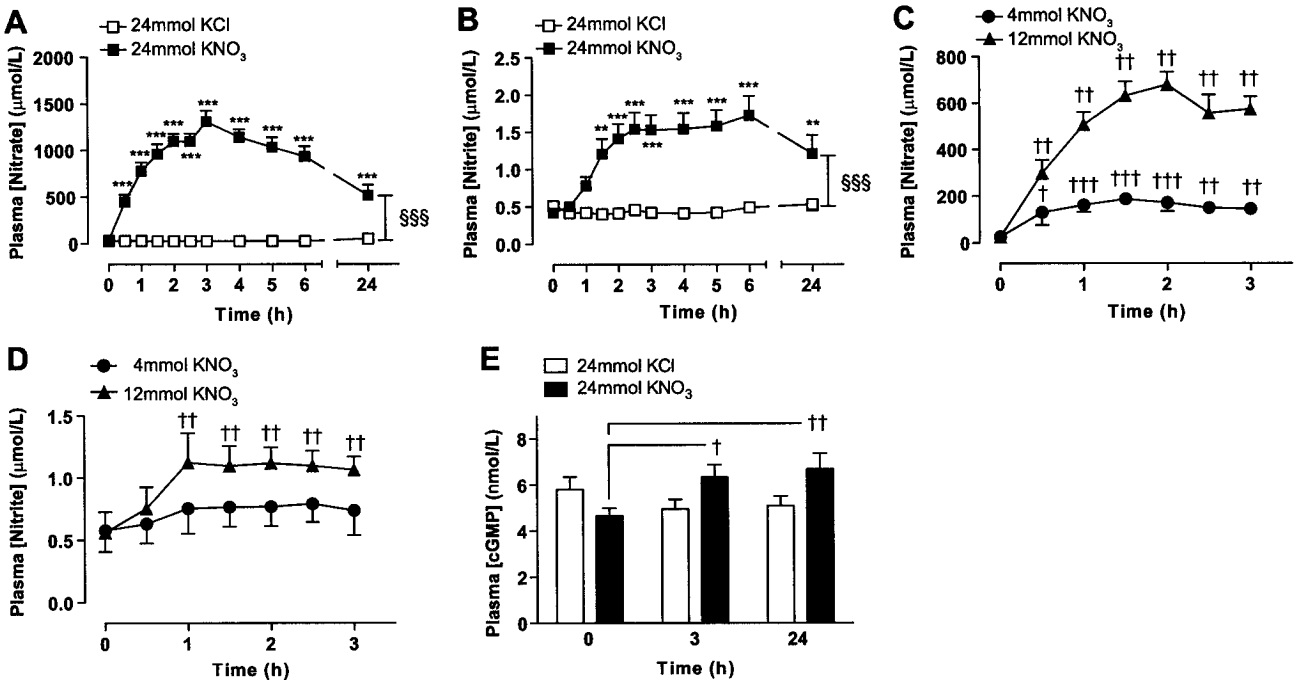

Figure 1. Dose-dependent effects of orally administered inorganic nitrate supplementation on plasma NOx. The effects of KNO_3 (24 mmol) and KCl (24 mmol) control capsules on circulating plasma (A) nitrate, (B) nitrite, and (E) cGMP (n=20); and the effects of 4 and 12 mmol of KNO_3 on circulating plasma (C) nitrate and (D) nitrite (n=6). Data are expressed as mean±SEM. Significance shown for comparisons between groups as §§§P<0.001 for 2-way ANOVA; **P<0.01 and ***P<0.001 for Bonferroni post hoc tests; and †P<0.05, ††P<0.01, and †††P<0.001 for 1-way ANOVA followed by Dunnett posttest comparison with baseline (t=0).

Dose-Dependent Decreases in BP After Oral Inorganic Nitrate Capsule Ingestion

KNO_3 (24 mmol) ingestion caused reductions in both SBP and DBP over 24 hours compared with KCl control. The peak differences between the 2 limbs were 9.4±1.6 mm Hg (at 6 hours) and 6.0±1.1 mm Hg (at 2.75 hours) for SBP and DBP, respectively (Figure 2A and 2B). There was no significant difference in the HR response between the 2 groups (Figure

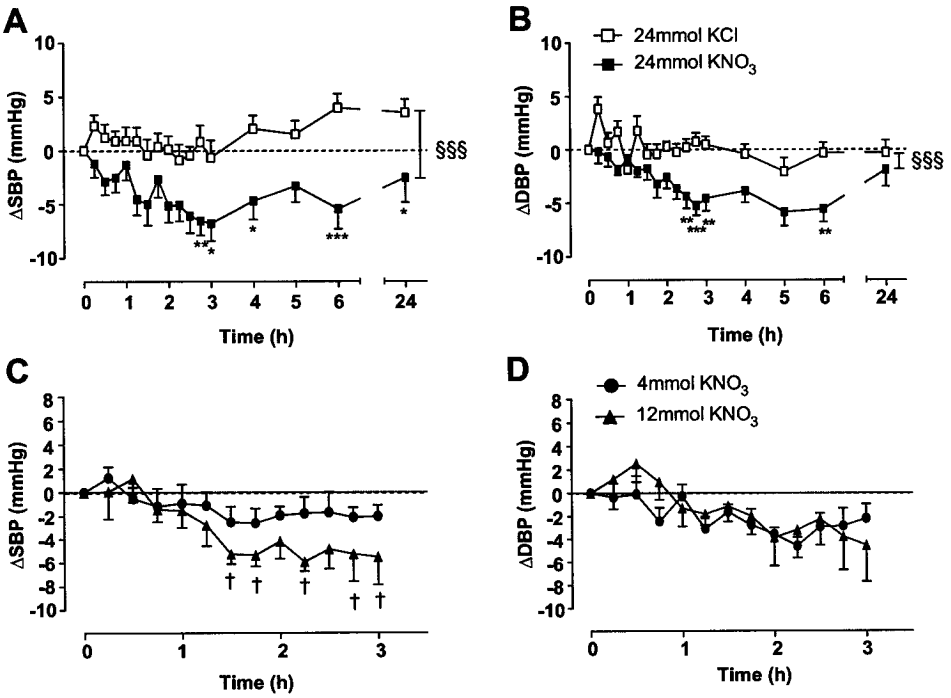

Figure 2. Inorganic nitrate supplementation lowers BP. The effects of KNO_3 (24 mmol) and KCl (24 mmol) on (A) SBP and (B) DBP (n=20) and the effects of 4 and 12 mmol of KNO_3 on (C) SBP and (D) DBP (n=6). Data are expressed as mean±SEM. Significance shown for comparisons between groups as §§§P<0.001 for 2-way ANOVA; *P<0.05, **P<0.01, and ***P<0.001 for Bonferroni post hoc tests; and †P<0.05 for 1-way ANOVA followed by Dunnett posttest comparison with baseline (t=0).

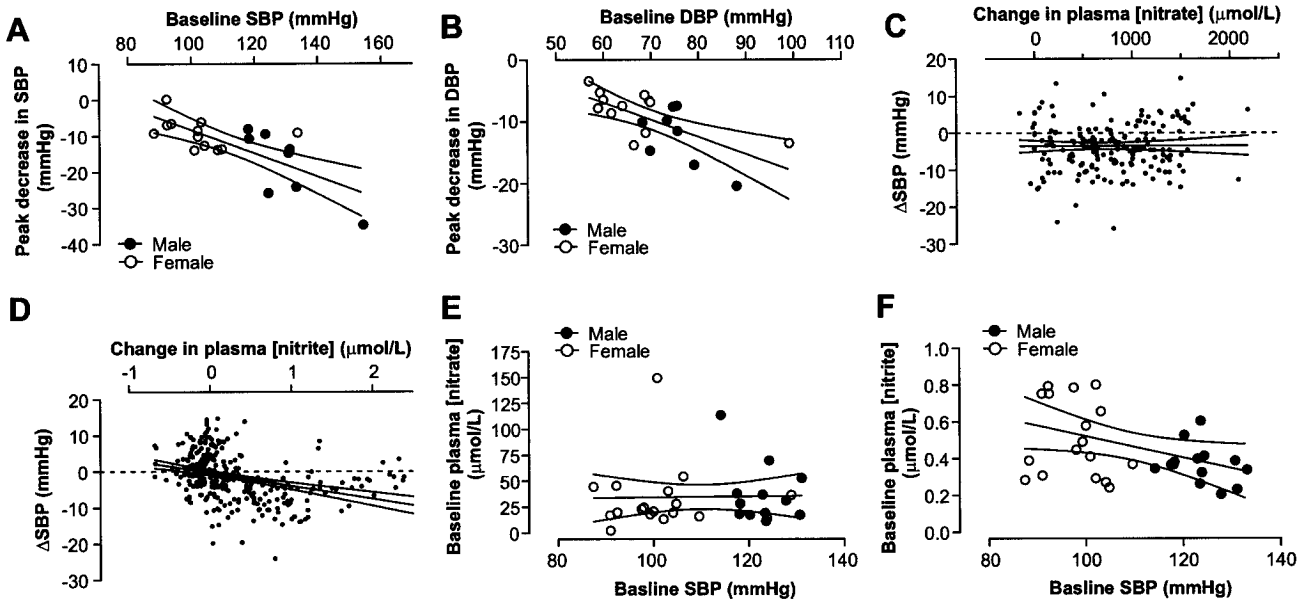

Figure 3. Plasma nitrite determines BP. Correlation of changes in (A) nitrate and (B) nitrite to changes in SBP, correlation of peak changes in (C) SBP and (D) DBP to baseline SBP, and correlation of baseline SBP to baseline (E) nitrate and (F) nitrite after KNO_3 or KCl (24 mmol) ingestion. All of the graphs show Pearson linear regression of best-fit ±95% CIs.

S1). The effect of KNO_3 on BP was dose dependent (Figure 2C and 2D).

Changes in SBP Correlate With Baseline BP and Plasma Nitrite But Not Nitrate Concentration

Post hoc analysis of the KNO_3/KCl capsule study demonstrated that the decreases in BP after nitrate ingestion are not correlated with changes in plasma nitrate concentration ($P=0.95$, linear regression; Figure 3A) but are correlated with changes in plasma nitrite concentration ($r=-0.350$; $P<0.001$, linear regression; Figure 3B). In addition, the peak decreases in BP are also correlated negatively with baseline BP (SBP: $r=-0.728$, $P<0.001$; DBP: $r=-0.657$, $P<0.01$; Figure 3C and 3D). Finally, baseline BP is correlated negatively with baseline nitrite ($r=-0.373$; $P<0.05$) but not nitrate ($P=0.93$; Figure 3E and 3F).

Sex Differences in Responses to Nitrate

Interestingly, the above post hoc correlations exposed a prominent sex difference in the responses to nitrate. Separation of the KNO_3/KCl capsule comparison study data by sex demonstrates that female volunteers had significantly lower baseline SBP, DBP, and body mass index (Table S2) compared with the male volunteers. In addition, whereas baseline plasma nitrate concentration was similar between the sexes, plasma nitrite concentration was significantly higher in the females (Table S2).

Additionally, the rise in plasma nitrate and nitrite concentration in males after KNO_3 ingestion appeared significantly lower compared with females (Figure 4A and 4B). However, the fold increases in plasma nitrite concentration from baseline were similar (≈3.3- and ≈4.1-fold for males and females, respectively). Conversely, KNO_3-induced reduction in SBP and DBP was substantially greater in males compared with females (Figure 4C and 4D). There were no significant effects on HR (Figure S1). No sex differences in the response to KCl with respect to SBP, DBP, or HR were found (Figure S2). The dose of nitrate per kilogram of body weight administered to females was 0.45±0.02 mmol/kg and for males was 0.32±0.021 mmol/kg (see Figure S3 for normalized plasma NOx relative to dose given).

Inorganic Nitrate Prevents IR-Induced Endothelial Dysfunction

In addition to the reduction in BP, nitrate capsules prevented IR-induced endothelial dysfunction (Figure S4). Moreover, this effect was not evident after chloride capsule ingestion.

Dose-Dependent Effects of Beetroot Juice

After juice ingestion (5.5 mmol nitrate dose), plasma nitrate rose rapidly and remained elevated over the 3-hour time course compared with water control (Figure 5A). Plasma nitrite concentration also increased, peaking at 2.5 hours with a ≈1.6-fold rise above baseline levels and also remaining significantly elevated over the 3-hour time course compared with water control (Figure 5B). In addition, cGMP levels were elevated at 3 hours compared with baseline after beetroot juice ingestion (Figure 5E). Although SBP decreased with a peak reduction of 5.4±1.5 mm Hg (SBP; Figure 5C) and endothelial dysfunction caused by IR injury prevented (Figure S3), there were no significant differences in DBP or HR between the limbs (Figures 5D and S1).

Discussion

Determining how vegetables confer protection against CVD and exploiting this to therapeutic advantage are likely to have considerable health and economic implications. Recently, it has been suggested that dietary nitrate found in high levels in vegetables might underlie some of the beneficial effects of vegetable-rich diets.[12,14] In the present study we have shown that inorganic nitrate capsules or a dietary nitrate load, in the

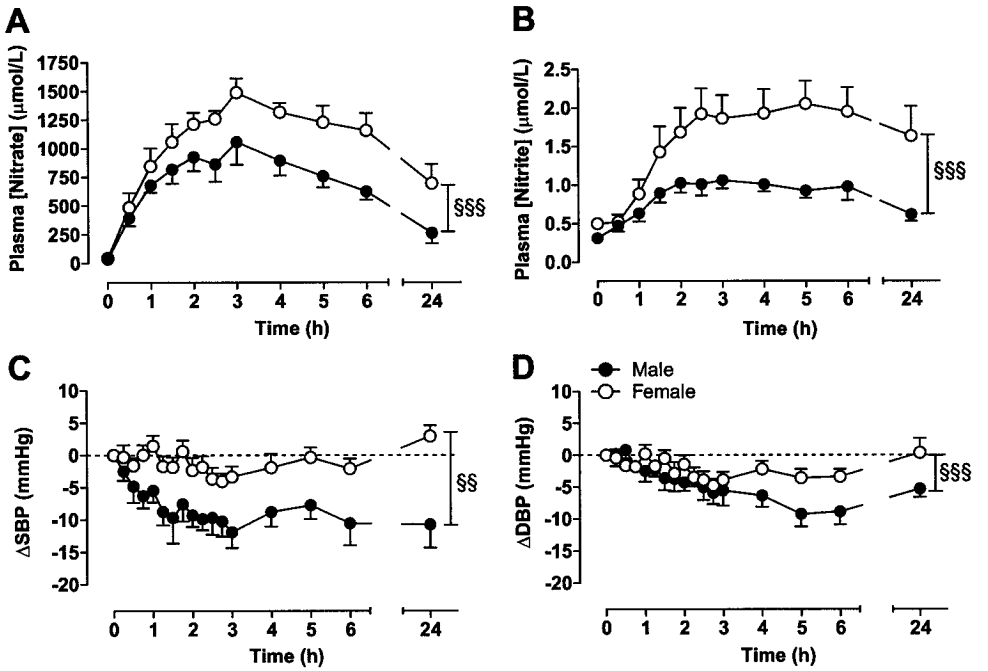

Figure 4. Sex differences in circulating plasma (A) nitrate and (B) nitrite and (C) SBP and (D) DBP after administration of KNO_3 (24 mmol) capsules. Data are expressed as mean±SEM of males (n=8) and females (n=12). Significance shown for comparisons between groups as §§P<0.01 and §§§P<0.001 for 2-way ANOVA.

form of beetroot juice, results in dose-dependent increases in plasma nitrite concentration via bioconversion in vivo. Stieglitz postulated,[20] >80 years ago, that the beneficial effects of inorganic nitrate (bismuth subnitrate) in hypertensive patients were because of conversion to nitrite in vivo, and our findings confirm that this bioactive nitrite, after reduction to NO, causes dose-dependent decreases in BP and prevents IR-induced endothelial dysfunction in healthy volunteers.

Ingestion of KNO_3 capsules caused rises in circulating plasma nitrate and thence nitrite concentration that were dose and time dependent. Significant rises in plasma nitrate concentration were evident sooner than nitrite (30 minutes versus

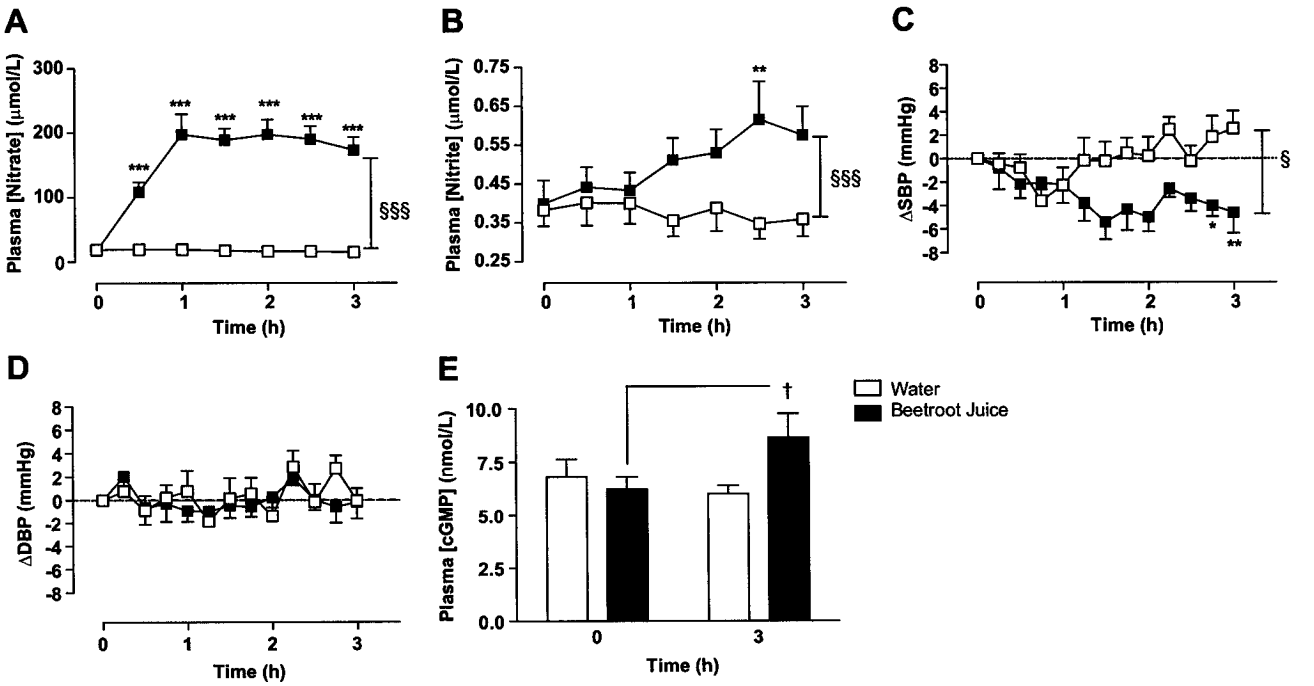

Figure 5. Dietary nitrate supplementation with beetroot juice raises plasma nitrite and lowers BP. The effects of beetroot juice (250 mL; 5.5 mmol of nitrate) or water control on circulating plasma (A) nitrate, (B) nitrite, (E) cGMP, (C) SBP, and (D) DBP. Data are expressed as mean±SEM of n=9. Significance shown for comparisons between groups as §P<0.05 and §§§P<0.001 for 2-way ANOVA; *P<0.05, **P<0.01, and ***P<0.001 for Bonferroni post hoc tests; and †P<0.05 for paired Student t test.

1.5 hours for 24 mmol of KNO_3) reflecting the use of the entero-salivary pathway and lingual bacterial reduction of nitrate to nitrite.[14,21] Approximately similar time courses for changes in both plasma nitrate and nitrite concentration were evident with lower doses of nitrate provided by either KNO_3 capsule or beetroot juice ingestion. Indeed, the dose of nitrate administered via beetroot juice of 5.5 mmol caused fold rises in plasma nitrate and nitrite concentration that fell between the effects of either 12 or 4 mmol provided via nitrate capsule. These findings indicate that, irrespective of source, that is, nitrate salt or in dietary form, the pharmacokinetics of nitrate and nitrite after an oral nitrate load remain largely unchanged and are dose dependent.

KNO_3 capsule ingestion substantially lowered SBP and DBP over 24 hours, whereas a similar dose of KCl did not alter BP over the same time period. These findings suggest that the BP changes were not attributed to the K^+ content and, more likely, dependent on the endogenous conversion to nitrite and, thence, to NO, because the changes in plasma nitrite correlated closely with reductions in BP. Nitrite, within the realm of physiological concentrations, vasodilates both the arterial and venous sides of the forearm circulation of humans,[17,22] and systemic nitrite application decreases BP in both primates[18] and humans.[17] In the main, it is thought that these effects of nitrite are because of its reduction to NO within the blood vessel wall[23,24] and within the red blood cell,[17,25] although there is some evidence that nitrite may exert direct effects independent of NO formation.[26,27] The effects of inorganic nitrate were found to be dose dependent as reflected by the decreasing magnitude of response in SBP to a 24-, 12-, 5.5-, and 4-mmol dose. Importantly, as with plasma NOx, this dose dependency appeared to hold irrespective of whether the inorganic nitrate load was administered by KNO_3 capsules or beetroot juice. The similarities between the activity of these 2 distinct approaches to inorganic nitrate administration are further reflected by the demonstration that KNO_3 capsules protect against the endothelial dysfunction induced by an IR insult much in the same manner as shown previously for a matched nitrate dose in beetroot juice.[14] This latter finding provides further support for our contention that inorganic nitrate underlies the beneficial effects of beetroot juice on the cardiovascular system.

Although it is largely accepted that NO underlies the bioactivity of nitrite, this has not been demonstrated clearly in humans in vivo. In the present study we demonstrate a temporal relationship between the rise in circulating nitrite concentration with a rise in cGMP levels. cGMP is the most sensitive indicator of NO bioactivity,[28] and evidence of its elevation provides unequivocal evidence of the generation of bioactive NO.

Post hoc analyses of the KNO_3 capsule data demonstrate that the magnitude of the BP response is directly related to baseline BP (ie, the higher the baseline BP the greater the peak BP reduction achieved). This relationship is consistent with the observation that the effect of BP-lowering drugs in patients is also proportional to resting BP.[29] Interestingly, in our cohort, baseline BP was closely correlated with baseline plasma nitrite but not nitrate concentration. Baseline plasma nitrite levels has been proposed to be an accurate reflection of endogenous NO generation via endothelial NO synthase–dependent conversion of L-arginine to NO,[30] and our findings may simply be highlighting the known relationship between classic NO synthase–derived NO and BP. However, with the appreciation that nitrite is a bioactive molecule, our findings also support the possibility that the correlation of baseline plasma nitrite with BP is actually a reflection of the functional activity of physiological nitrite reduction as first proposed in 2000 by Gladwin et al.[31] This, in turn, raises the possibility that intrinsic plasma nitrite concentration may be involved in "setting" the BP of healthy volunteers. Interpretation of plasma NOx is challenging because of the fact that multiple pathways for the generation and destruction of NOx and NO exist.[19] Indeed, changes in plasma nitrite concentration may reflect endothelial NO synthase activity,[30] NO oxidation,[32] nitrate reduction,[14,21] or all 3 at once. Nevertheless, plasma nitrite concentration correlated with BP at baseline and with changes in BP after nitrate supplementation, with corresponding increases in plasma cGMP concentration, suggesting that the measure of plasma nitrite does reflect, at least in part, nitrite bioactivity. Our data also appeared to suggest some clustering of responsiveness to nitrate into 2 groups, that is, although small changes in nitrite ($\approx<1$ μmol/L) effected apparently substantial changes in BP, where the changes in nitrite were >1 μmol/L, little effect on BP was evident. Further post hoc analyses of our data suggest that, indeed, 2 distinct groups of responsiveness to inorganic nitrate dosing exist within our cohort according to sex.

A significant difference in baseline plasma nitrite concentration (but not plasma nitrate) associated with lower baseline BP was evident in our female volunteers compared with the male volunteers. This finding is supportive of the view that a close relationship between nitrite levels and BP exists in humans. This correlation has been demonstrated previously but attributed to differences in vascular endothelial NO synthase expression and activity,[33] an effect that, in addition to endothelium-derived hyperpolarizing factor,[34] has been proposed to mediate the prevalence of lower BP in premenopausal women compared with age-matched men.[35] Our data herein also raise the further possibility that the association of circulating nitrite levels with lower BP evident in premenopausal women may relate, in part, to the bioactivity of the elevated levels of nitrite. This difference in basal nitrite levels may underlie the apparent decreased sensitivity to further elevations in plasma nitrite concentration. Dejam et al[18] have demonstrated that, whereas low micromolar concentrations of nitrite produced substantial increases in blood flow in the forearm, a saturation of the vasodilatory effect was observed with higher micromolar levels. It is possible that the apparent lack of effect of nitrite in females relates to a similar "saturation" of its vasodilating effect.

In addition, our analyses intimate sex differences in the endogenous handling of nitrate. Indeed, a similar dietary nitrate load, whilst resulting in only subtle differences in nitrate levels between the sexes, caused a $\approx$2-fold higher plasma nitrite concentration in females compared with males. These data hint at intriguing sex differences in the processing of NOx. Although differences in absorption and excretion of NOx may underlie some of these differences, it is possible

that the differences in nitrite levels reflect different lingual bacterial loads or species responsible for nitrate reduction to nitrite. Currently, it is thought that the predominant lingual bacteria responsible for nitrate reduction is Gram-negative *Veillonella spp* and Gram-positive *Actinomyces spp*.[36] Whether sex differences exist in the colonization of the tongue or nitrate reductase activities of these bacteria is currently unknown. It is also possible that the differences in plasma NOx levels simply reflect the differences in body weight between the 2 sexes, which were greater in the females compared with the males. However, normalization of plasma NOx concentrations to body weight did not alter the shape of the profiles seen, and significant differences in plasma nitrite concentration still persisted.

Taking all of the post hoc analyses together, we suggest that these apparent differences in processing of nitrate are likely to contribute to the prevalence of lower baseline BP in women compared with men.[35] An important limitation of our findings is that the sex differences were exposed with post hoc analyses. Further investigation in a prospective fashion to corroborate these analyses is clearly warranted. In addition, we did not control for the stage of the menstrual cycle in our female volunteers, and this may have some relevance, because resting BP is different throughout the menstrual cycle.[37]

Finally, in all of the measures of bioactivity, no significant changes were observed in the control limb using KCl capsules to match the 24-mmol KNO_3 dose. The significance of this finding is 2-fold. First, this suggests that the effects on BP were attributable specifically to the activity of nitrate. Secondly, the lack of any BP effect of KCl also supports the view that, whilst potassium (dietary or supplementation[38]), known to exert a number of beneficial effects on the cardiovascular system, particularly decreases in BP, it is not responsible for the effects of KNO_3 supplementation and is unlikely to underlie the effects of beetroot juice.

Perspectives

Although we acknowledge that our studies represent the responses of a healthy volunteer population, our evidence suggests that a dietary nitrate approach to CVD may have therapeutic use. This view is supported by the fact that the dose of 24 mmol administered in this study roughly approximates to the estimated nitrate content (≈20 mmol)[39] in the Dietary Approaches to Stop Hypertension diet,[9] a diet associated with significant decreases in BP. Extrapolation of the beneficial effects of dietary (inorganic) nitrate to the wider population, including patients with CVD, will require large-scale outcome trials to prove the thesis that dietary (inorganic) nitrate is a potential preventative measure or treatment for CVD. Furthermore, we suggest that important sex differences in baseline levels and handling of NOx species may underpin differences in BP and CVD in the general population. Finally, there may be a role for nitrate in delaying and preventing hypertension, and supplementation either in water or by diet may provide a cheap and effective global health strategy to combat the prevalence of CVD.

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Disclosures

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Correction

In the version of the *Hypertension* article "Inorganic Nitrate Supplementation Lowers Blood Pressure in Humans: Role for Nitrite-Derived NO" by Kapil et al that was posted online on June 28, 2010 (DOI: 10.1161/HYPERTENSIONAHA.110.153536), an error occurred.

In the *y*-axis labels for Figures 1B and D, 3D and F, 4B, and 5B, "Nitrate" should be "Nitrite."

These corrections have been included in the final print version of the article in the August 2010 issue of the journal (*Hypertension.* 2010;56:274–281) and in the current online version, which is available at http://hyper.ahajournals.org/cgi/content/full/56/2/274. The corrected figures appear below.

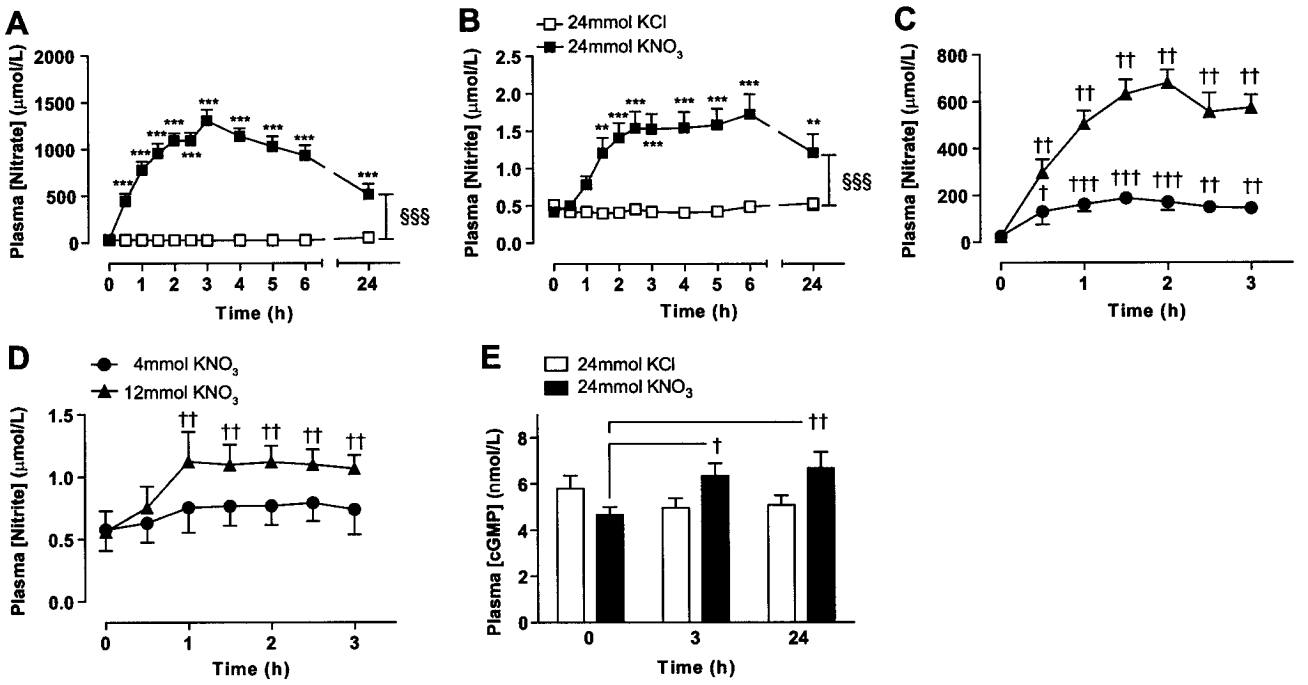

Figure 1.

(*Hypertension.* 2010;56:e37-e39.)

Hypertension is available at http://hyper.ahajournals.org

DOI: 10.1161/HYP.0b013e3181f01f25

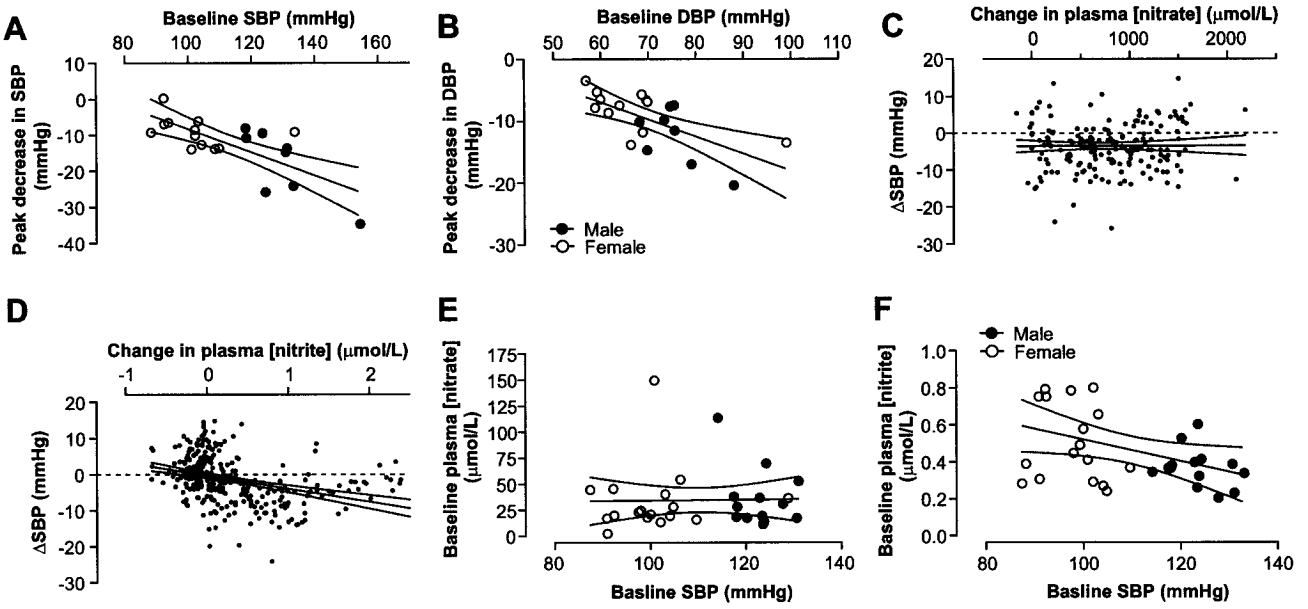

Figure 3.

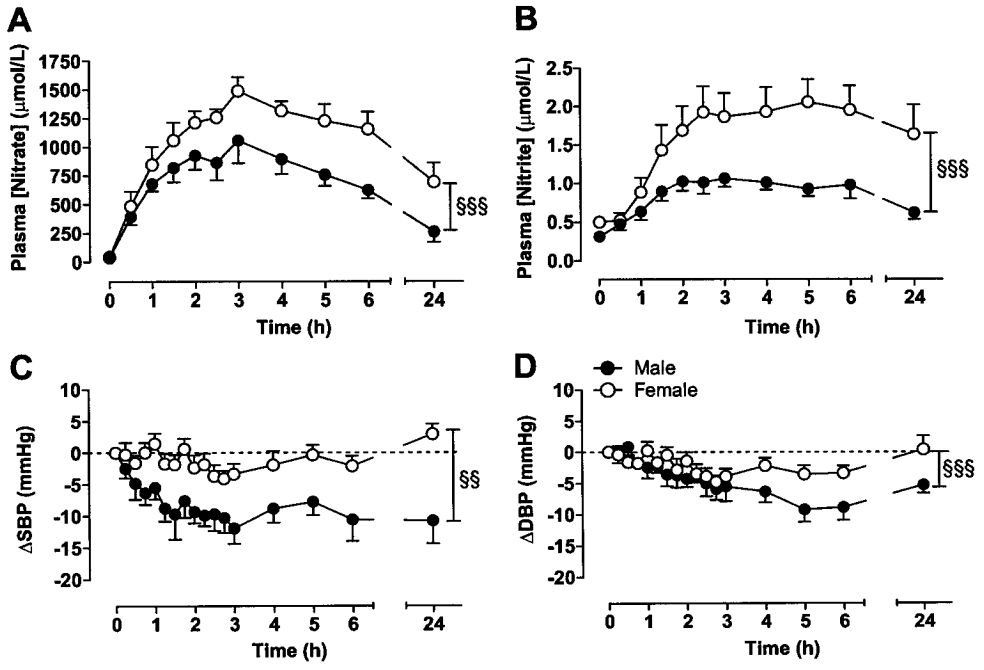

Figure 4.

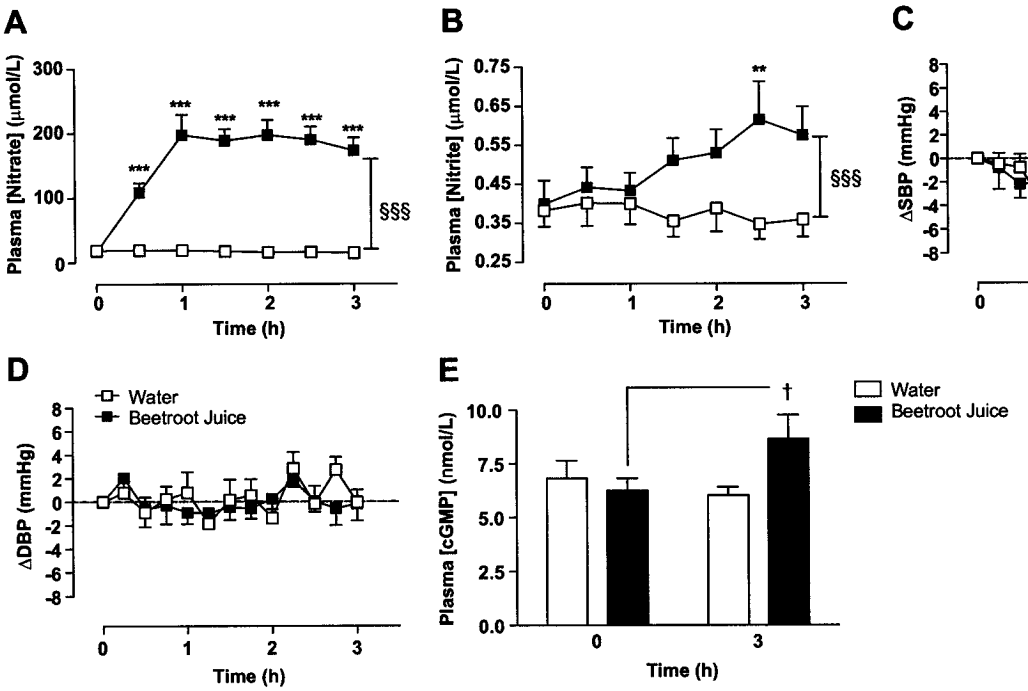

Figure 5.

Hypertension regrets the error.

Online supplement

Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived nitric oxide

Vikas Kapil[1], Alexandra B Milsom[1], Michael Okorie[2,3], Sheiva Maleki-Toyserkani[1], Farihah Akram[1], Farkhanda Rehman[1], Shah Arghandawi[1], Vanessa Pearl[1], Nigel Benjamin[4], Stavros Loukogeorgakis[2,3], Raymond MacAllister[2], Adrian J Hobbs[5], Andrew J Webb[1], Amrita Ahluwalia[1]

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[2]Centre for Clinical Pharmacology, University College London, UK;

[3] University of Exeter and Plymouth, Exeter, UK;

[4] Vascular Physiology Unit, Institute of Child Health, University College London, London, UK;

[5]Neuroscience, Physiology and Pharmacology, University College London, UK.

Methods Supplement

Volunteers

The studies were granted full ethical approval by the Local Research Ethics Committee. All subjects gave informed consent after satisfying the inclusion criteria: healthy male or female adult of 18-45 years of age, a BMI of 18-40 kg/m^2, no systemic medication (other than the oral contraceptive pill) and non-smoker. Patients were instructed to keep to a low nitrate diet (i.e. no processed meat or green leafy vegetables) and to abstain from strenuous exercise on the day preceding the study and were fasted overnight. On arrival, volunteers were provided with a light breakfast (slice of dry wholemeal toast). There were 3 distinct phases of volunteer recruitment, each phase requiring between 2-4 visits with a minimum of 7 days between each visit.

Flow mediated dilatation (FMD)

In a temperature-controlled environment (24-26°C), endothelial function was assessed by measuring brachial artery diameter in response to reactive hyperemia as previously described.[1] A B-mode scan of the brachial artery was obtained in longitudinal section above the antecubital fossa using a 7.0-MHz linear array transducer and a standard Acuson XP10 system (Acuson, Mountain View, California). Arterial diameter over a 1-2-cm section was determined for each image with the use of automatic edge-detection software (Brachial Tools, Iowa City, Iowa). Blood flow was manipulated in the brachial artery by a 7-cm-wide blood pressure cuff placed around the forearm immediately below the antecubital fossa. After 1 min of baseline flow, the cuff was inflated to 300 mm Hg for 5 min and released, resulting in a brief episode of reactive hyperemia. Brachial artery diameter changes in response to blood flow were assessed for a further 5 min. To determine the effect of IR on endothelial function, FMD was assessed before ischemia (induced by inflating a BP cuff placed around the upper part of the arm to a pressure of 200 mmHg for 20 min) and following 20 min reperfusion (achieved by cuff deflation).[1,2] Brachial artery diameter was measured in millimeters and dilation expressed as percentage increase from baseline diameter.

Blood sampling

A 19-gauge butterfly needle, with extension set, was inserted prior to capsule or juice ingestion, and if required again, at 24h, and secured to skin. Blood samples were taken at baseline and then, following capsule, beetroot juice or water ingestion, every 30 min up to 3h, and then in some studies hourly from 3-6h and then again at 24h. Blood samples were taken atraumatically, via the butterfly needle, into pre-chilled lithium heparin tubes and immediately spun at 1300G at 4°C for 10 min. Plasma was separated and stored at -80°C until measurement of [nitrate] and [nitrite] were undertaken.

Blood pressure measurement

All BP and heart rate (HR) measurements were taken in triplicate in the seated position using an Omron 715IT. Subjects and investigators were blinded to the readings by means of laminated coverings for the machine and printer. The mean of the 2nd and 3rd readings were used for analysis purposes. BP was measured every 15 min for 1h to establish a baseline BP. Following capsule or beetroot juice ingestion BP measurements were taken every 15 min for

3h, then for those studies of duration longer than 3h, hourly for a further 3h and finally at 24h.

Measurement of plasma nitrate/nitrite

Prior to ozone chemiluminescence, plasma samples were filtered using Microcon® Ultracel YM-3 (3 kDa) filters (Millipore Corporation, Billerica, USA) and then [nitrate] and [nitrite] in the filtrate determined as previously described. [3] Briefly, samples and standards containing nitrite and nitrate were first reduced to NO, which was then quantified using a NO analyzer (NOA 280, Sievers, Boulder, USA). To determine total [nitrite] and [nitrate] (NOx), samples were added to 0.1 mol/L vanadium (III) chloride in 1M hydrochloric acid refluxing at 90°C under nitrogen. Nitrite concentrations were determined by addition of samples to 1.5 % potassium iodide in glacial acetic acid under nitrogen at room temperature. Concentrations of nitrate were calculated by subtraction of [nitrite] from NOx values.

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Tables

Table S1 Volunteer demographics and baseline hemodynamic parameters for the 3 distinct BP studies and FMD study. Data are shown as mean ± SEM values. Baseline BP = mean of readings in 1st hour. ND = not determined.

Study	KNO_3 vs. KCl	KNO_3 dose-response	Beetroot juice	FMD
Subjects (n)	20	6	9	12
Age (years)	22.5±0.9	28.8±1.7	25.1±1.1	24.7±1.4
BMI (kg/m^2)	22.5±0.6 (range 19.6-30.0)	24.5±1.6 (range 18-29.3)	26.5±0.9 (range 23.1-30.7)	ND
Baseline SBP (mmHg)	110.1±3.4	114.5±4.6	120.6±4.1	103.6±0.8
Baseline DBP (mmHg)	70.1±2.3	71.0±2.2	70.9±2.5	62.6±0.7

Table S2 Sex differences in demographics, baseline hemodynamic characteristics and baseline plasma [nitrate] / [nitrite] for 24mmol inorganic nitrate vs. chloride control study. Significance values for unpaired Student t-test shown in last column.

Baseline Characteristics	Male (n=8)	Female (n=12)	Significance
Age (years)	23.0±1.4	22.3±1.2	p=0.70
BMI (kg/m^2)	24.2±1.0	21.4±0.7	p<0.05*
SBP (mmHg)	126.4±2.5	101.5+2.3	p<0.001***
DBP (mmHg)	73.3±1.4	66.7±2.2	p<0.05*
Plasma [Nitrate] μM	35.0±6.9	33.8±7.9	p=0.91
Plasma [Nitrite] μM	0.362±0.03	0.536±0.05	p<0.01**

Statistical significance shown as * for p<0.05, ** p< 0.01 and *** for P<0.001 using unpaired students t-test.

Supplementary Figures

Figure S1

Inorganic or dietary nitrate supplementation does not significantly alter heart rate (HR). The effects on change in HR after administration of (A) 24mmol KNO_3 and KCl (n=20), (B) 4mmol and 12mmol KNO_3 (n=6), (D) beetroot juice (250 ml; 5.5 mmol nitrate) (n=9); and (C) sex-differences in change in HR from baseline after administration of 24mmol KNO_3 (males n=8, females n=12). Data are expressed mean ± SEM. No significant differences between groups following 2-way ANOVA.

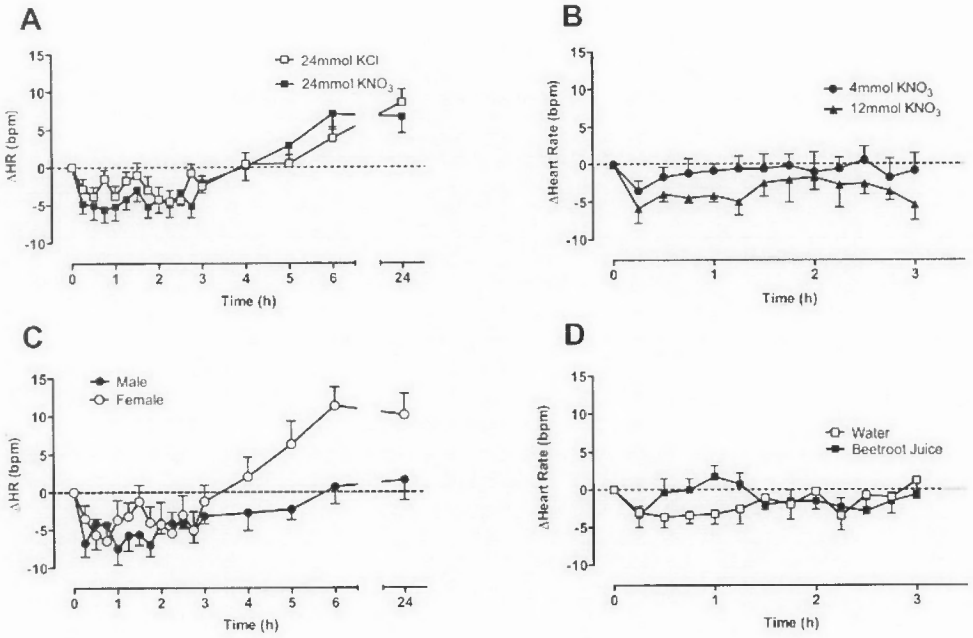


Figure S2

Sex differences in change in (A) SBP, (B) DBP and (C) HR from after administration of KCl (24mmol) capsules. Data are expressed as mean ± SEM of males n=8 and females n=12. No significant differences between groups following 2-way ANOVA.

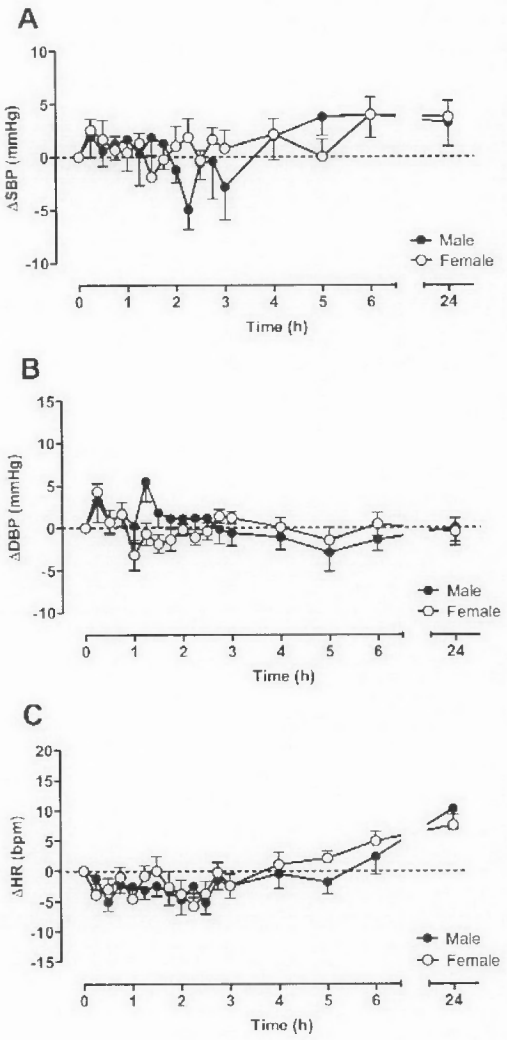


Figure S3

Plasma [nitrate]/[nitrite] relative to dose administered following 24mmol KNO_3 administration. Data are expressed mean ± SEM. Significance shown for comparisons between groups as §p<0.05, §§§p<0.001 for 2-way ANOVA.

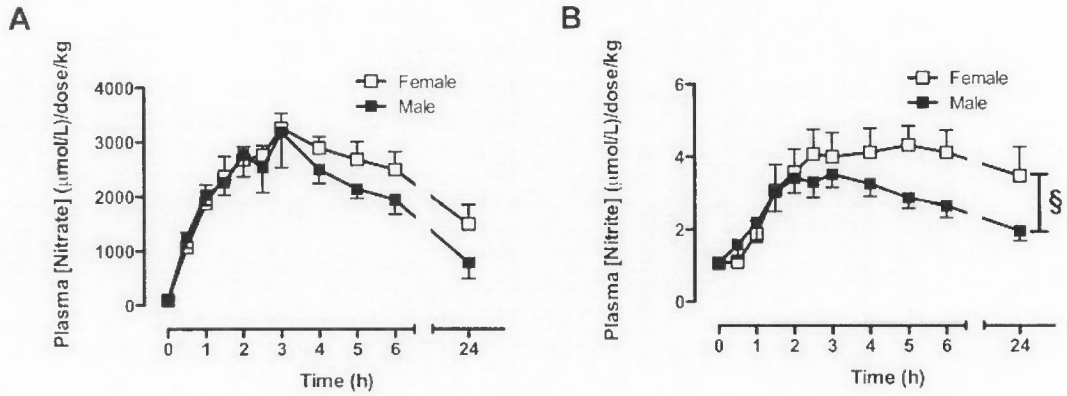
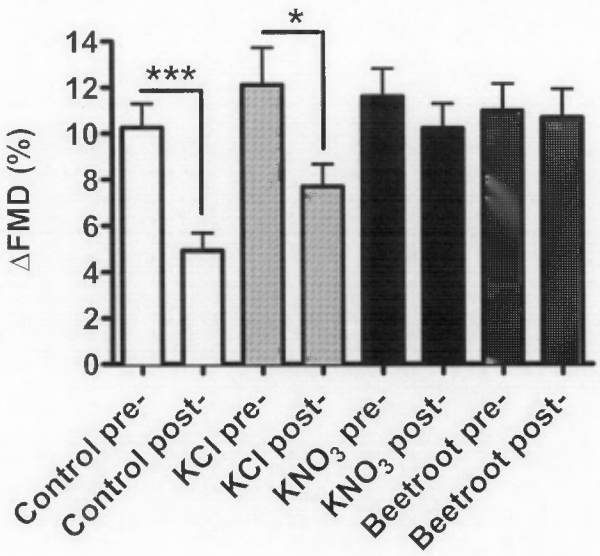


Figure S4

Inorganic nitrate ingestion protects against IR-induced endothelial dysfunction. FMD (%) after IR injury (control pre- and post-) and after administration of KCl capsules (24mmol), KNO_3 capsules (24mmol) or 250ml beetroot juice (5.5mmol). Data are expressed mean ± SEM of n=12. Significance shown as *p<0.05 and ***p<0.001 for Bonferroni post-hoc tests, following 1-way ANOVA, for comparisons between groups.



Correction

In the version of the *Hypertension* article "Inorganic Nitrate Supplementation Lowers Blood Pressure in Humans: Role for Nitrite-Derived NO" by Kapil et al that was posted online on June 28, 2010 (DOI: 10.1161/HYPERTENSIONAHA.110.153536), an error occurred.

In the *y*-axis labels for Figures 1B and D, 3D and F, 4B, and 5B, "Nitrate" should be "Nitrite."

The corrections have been made in the current online version and will be made in the final print version of the article in the August 2010 issue of the journal.

Hypertension regrets the error.

The authors reply:

To the Editor: We agree with Dr. Redberg's comments. Although much effort has recently been directed toward controlling health care costs,[1] little attention has been given to prevention as a method of cost containment. Our findings indicate the potential for preventive measures to increase the proportion of the population with favorable cardiovascular risk-factor profiles, thereby preventing cardiovascular disease and lowering health care costs. Such a result requires population-wide early prevention of all major risk factors (rather than merely the treatment of existing risk factors and disease). This critical conclusion was overlooked in the editorial, which focused on expensive drug treatments for persons at high risk.

In her editorial, Dr. Russell casts doubt on our ability to achieve very early modification of risk factors, and she argues that intensive, multiple-intervention programs would be costly.[2] In fact, during the past two decades, relatively inexpensive preventive efforts have achieved sizable reductions in the mean intakes of saturated fat and cholesterol, in serum cholesterol (from about 240 to less than 205 mg per deciliter), in blood pressure, and in the prevalence of smoking — resulting in a reduction of more than 50 percent in the mortality from coronary heart disease or other forms of cardiovascular disease.[3] Our data also show graded increases in Medicare charges, with charges rising progressively with the level of each major cardiovascular risk factor.[3] All these findings support the inference that public policy efforts to shift behavior toward favorable risk-factor patterns result in less disease and lower Medicare costs, even before large numbers of people achieve low-risk status (defined as freedom from all major risk factors).

With respect to the issue raised by Gaenzer et al., the mean annual Medicare charges — both total charges and those related to cardiovascular care — were significantly lower for people with a favorable body-mass index (<25) than for those with a higher body-mass index, after we controlled for all other risk factors ($531 vs. $557 for men, and $333 vs. $433 for women).

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1. Schwartz WB. The inevitable failure of current cost-containment strategies: why they can provide only temporary relief. JAMA 1987;257:220-4.
2. Russell LB. Prevention and Medicare costs. N Engl J Med 1998;339:1158-60.
3. Lowe LP, Liu K, Daviglus M, et al. CVD risk factors and health care costs 20+ years later: the CHA Study. Can J Cardiol 1997;13:Suppl B:31B. abstract.

To the Editor: Dr. Redberg may be right about insurers, but cost-effectiveness analyses take a long-term view and already account for the kind of future savings documented by Daviglus et al. These analyses also account for the costs of the interventions necessary to reap those savings, something done by neither Dr. Redberg nor Daviglus et al. Thus, their data do not change the problem or the information available to policy makers in the Health Care Financing Administration and elsewhere; since the cost effectiveness of the various interventions against heart disease (especially smoking cessation and medications to lower blood pressure and cholesterol levels) is highly variable, policy makers need to think carefully about the best ways to allocate resources for better health.

Dr. Redberg does not cite studies to support her statement that exercise and diet do not involve cost or risk, nor do I know of any that support that view. I have described elsewhere a framework for conducting an evaluation of exercise.[1] It is important to keep in mind that interventions that may not involve costs to the medical sector usually have costs outside that sector. Time, for example, is a scarce resource, and exercise requires a regular amount of it over the course of many years. Such steady, repeated costs for large populations make interventions more expensive than they first appear. To support good decisions about the best ways to improve health, information about costs and health benefits should be comprehensive, not limited to the issues of concern to a single sector.

Although I did not, as Dr. Redberg asserts, advocate screening for elevated homocysteine levels, I did advocate a search for more cost-effective interventions against heart disease. Homocysteine has been identified as a significant risk factor for heart disease. If interventions aimed at lowering homocysteine levels are evaluated in clinical trials, their cost effectiveness should be evaluated at the same time.

It is unfortunate that public health advocates continue to point to the savings from interventions while ignoring their costs. Good health is so important that we need to spend our resources as wisely as possible in its pursuit. The false claim that no resources are really required does not contribute to good decisions by policy makers or by individual men and women.

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Interstitial Nephritis in a Patient Taking Creatine

To the Editor: Creatine is being widely used as a possible performance-enhancing substance. Its safety, however, is being debated in both the scientific community[1-3] and in the popular press. We report on a patient who had transient renal insufficiency while taking creatine.

A previously healthy 20-year-old man presented with a four-day history of nausea, vomiting, and bilateral flank pain that began approximately four weeks after he started taking 5 g of creatine (pure creatine monohydrate, Pro-Performance Laboratories, Pittsburgh) orally four times a day. He had stopped the creatine and had not taken any medications or other food supplements. His blood pressure was 140/90 mm Hg. Physical examination revealed dehydration and diffuse abdominal tenderness. His serum creatinine concentration was 1.4 mg per deciliter (124 μmol per liter). A complete blood count and measurements of antistreptolysin O, antinuclear antibodies, and plasma complement C3 and C4 concentrations were nor-

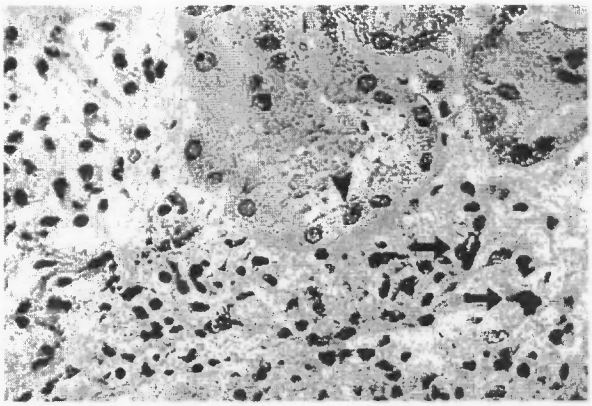

Figure 1. Electron Micrograph of a Renal-Biopsy Specimen Showing Acute Focal Interstitial Nephritis.

Shown are a proximal tubule with flattened epithelium (arrowhead) and an interstitial mononuclear infiltrate with interspersed eosinophils (arrows) (hematoxylin and eosin, ×256).

mal. Urinalysis revealed 4+ protein and 1+ blood; the urine sediment contained dysmorphic red cells and white-cell casts. A spiral computed tomographic scan revealed no abnormalities in the kidneys or the collecting systems.

The patient was hospitalized and treated with intravenous fluid and pain medication. During hospitalization, his blood pressure rose to 160/100 mm Hg and his serum creatinine concentration rose to a peak value of 2.3 mg per deciliter (203 μmol per liter). Urinary protein excretion was 472 mg per day. A renal biopsy revealed acute focal interstitial nephritis and focal tubular injury (Fig. 1); electron microscopy revealed effacement of glomerular foot processes and focal thickening of the basement membrane. The patient's blood pressure, serum creatinine concentration, and urinalysis subsequently became normal.

This case of acute interstitial nephritis should serve as a warning that the use of creatine, which is freely available in stores, may be associated with renal injury.

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1. Pritchard NR, Kaira PA. Renal dysfunction accompanying oral creatine supplements. Lancet 1998;351:1252-3.
2. Greenhaff P. Renal dysfunction accompanying oral creatine supplements. Lancet 1998;352:233-4.
3. Poortmans JR, Francaux M. Renal dysfunction accompanying oral creatine supplements. Lancet 1998;352:234.

A spokesperson for General Nutrition Products replies:

To the Editor: General Nutrition Products packages creatine under the ProPerformance label for General Nutrition Centers. The inference that creatine is unsafe for use as a dietary supplement should be tempered in view of the large and growing body of scientific literature on creatine, the wide popularity of creatine as a performance-enhancing dietary supplement among athletes, and the lack of data from controlled studies that would support a causal relation between creatine and renal disease.

Creatine has been studied for many years as a performance-enhancing supplement. A majority of the studies show beneficial effects on performance during intense athletic activity.[1-3] These studies, as well as those that did not report a benefit,[4,5] are consistent in that they do not report major side effects among the subjects.

In these studies, creatine intake varied, but most subjects used some type of creatine-loading technique, with 20 g of creatine per day for five days a not unusual regimen.[3] In none of these studies did the subjects take 20 g per day for 30 days, the regimen described in the case report. The product sold by General Nutrition Centers that was taken by the subject in the case report has clearly written directions for use on the label that read in part, "Mix one heaping teaspoon (5 g) in your favorite beverage daily. For creatine loading, take four heaping teaspoons per day: one heaping teaspoon at four-hour intervals. Continue this process for four days. Do not exceed the loading level of more than four days in any one-month period."

General Nutrition Centers is committed to offering the public safe food supplements that provide benefits to the consumer. We are also committed to labeling products clearly to assist the consumer in the safe use of such products. We believe that creatine is both safe and beneficial, as demonstrated by the majority of the data on its use in the medical and scientific literature.

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Detection of Liver Masses with Spiral Computed Tomography

To the Editor: A recently developed technique, spiral computed tomographic (CT) scanning, may be particularly useful in the identification of liver masses. Data indicate that hepatocellular carcinomas and other liver masses with a predominantly arterial blood supply are best detected during the arterial phase of contrast enhancement, which occurs between 20 and 40 seconds after the administration of intravenous contrast material.[1,2] Spiral CT scanning

Long-Term Use of Short- and Long-Acting Nitrates in Stable Angina Pectoris

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Abstract: Long-acting nitrates are effective antianginal drugs during initial treatment. However, their therapeutic value is compromised by the rapid development of tolerance during sustained therapy, which means that their clinical efficacy is decreased during long-term use. Sublingual nitroglycerin (NTG), a short-acting nitrate, is suitable for the immediate relief of angina. In patients with stable angina treated with oral long-acting nitrates, NTG maintains its full anti-ischemic effect both after initial oral ingestion and after intermittent long-term oral administration. However, NTG attenuates this effect during continuous treatment, when tolerance to oral nitrates occurs, and this is called cross-tolerance. In stable angina long-acting nitrates are considered third-line therapy because a nitrate-free interval is required to avoid the development of tolerance. Nitrates vary in their potential to induce the development of tolerance. During long-lasting nitrate therapy, except pentaerythritol tetranitrate (PETN), one can observe the development of reactive oxygen species (ROS) inside the muscular cell of a vessel wall, and these bind with nitric oxide (NO). This leads to decreased NO activity, thus, nitrate tolerance. PETN has no tendency to form ROS, and therefore during long-term PETN therapy, there is probably no tolerance or cross-tolerance, as during treatment with other nitrates.

Key Words: Stable angina pectoris, organic nitrates, nitrate tolerance, nitrate cross-tolerance.

INTRODUCTION

Organic nitrates, apart from beta-blockers and calcium antagonists, are the mainstays of antianginal drug therapy in patients with stable angina pectoris. These nitrates are considered as valuable symptomatic agents, improving the quality of life of patients with angina. Although, they are effective antianginal drugs during initial treatment, their therapeutic value is compromised by the rapid development of tolerance during sustained therapy. Thus, their major disadvantage is connected with the occurrence of tolerance, which means that their clinical efficacy is decreased during long-term use. There is widely accepted opinion, that all patients with angina should receive a prescription for sublingual nitroglycerin (NTG) and instructions on its use [1,2]. However, long-term use of long-acting nitrates in stable angina pectoris is much more controversial [1,2]. Therefore, the pharmacological and physiological benefits of nitrates in coronary artery disease, and also some their potentially harmful mechanisms are discussed in this paper.

MECHANISM OF ANGINA PECTORIS

Myocardial ischemia, which produces angina pectoris, the principal symptom of coronary heart disease, results from a relative lack of coronary blood flow, generally due to partial obstruction of a large coronary artery. Atherosclerotic fixed obstruction is often worsened in a given patient by superimposed spasm, whereas collateral coronary blood flow into the ischemic area is variable from time to time as well as from patient to patient [2]. Both the typical and variant (vasospastic or Prinzmetal's) forms of angina may be manifested by sudden, severe, pressing substernal pain that radiates to the left shoulder and along the flexor surface of the left arm. However, the location and character of chest pain may vary [3].

Typical angina pectoris (stable angina) is commonly induced by exercise, emotion, or eating and is often associated with depression of the ST segment of the electrocardiogram (ECG). Typical angina is usually due to advanced atherosclerosis of the coronary vasculature. In contrast, variant angina is caused by vasospasm of the coronary vessels and may not be associated with severe atherosclerosis. Patients with Prinzmetal's angina may develop chest pain while at rest and exhibit elevation of the ST segment of the ECG [4].

Anginal attacks may recur for years or may rapidly increase in their frequency (unstable angina). They result from temporary ischemia of the myocardium, such that blood flow is insufficient to maintain adequate oxygenation. This can be due to a decrease in myocardial blood flow, an increase in the requirement of the myocardium for oxygen, or both. Angina is caused by myocardial ischemia, which occurs whenever myocardial oxygen demand exceeds oxygen supply. An understanding of the pathophysiology of angina first requires a brief review of the determinants of oxygen demand and supply [3].

There are four major factors that determine myocardial work and therefore myocardial oxygen demand (Table **1**).

Clinical conditions associated with an increase in oxygen demand must affect one or more of these parameters. Examples include increased catecholamines, as with vigorous

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1574-8847/09 $55.00+.00

Table 1. Factors that Determine Myocardial Work and Therefore Myocardial Oxygen Demand [3]

1.	Heart rate
2.	Systolic blood pressure (the clinical marker of afterload)
3.	Myocardial wall tension or stress (the product of ventricular end-diastolic volume or preload and myocardial muscle mass)
4.	Myocardial contractility

exertion or mental stress [5], tachycardia of any etiology, hypertension, and left ventricular hypertrophy.

Myocardial contractility and wall stress cannot be measured clinically. As a result, myocardial oxygen demands are estimated clinically by the multiplication product (also called the double product) of the heart rate and the systolic blood pressure. Individuals reproducibly experience angina during exercise testing when asked to exceed a well defined angina threshold or absolute double product value.

The major determinants of myocardial oxygen supply are the oxygen carrying capacity of the blood, which is affected by a variety of factors including oxygen tension, the hemoglobin concentration; the degree of oxygen unloading from hemoglobin to the tissues, which is related to 2,3 diphosphoglycerate levels; and the coronary artery blood flow. Coronary blood flow is influenced by the following factors (Table **2**).

Table 2. Factors that Influence Coronary Blood Flow [6,7]

5.	Coronary artery diameter and tone (resistance) [6,7].
6.	Collateral blood flow.
7.	Perfusion pressure, which is determined by the pressure gradients from the aorta to the coronary artery and, since flow is from epicardium to endocardium, from the coronary artery to the endocardial capillaries. The flow within the endocardium is determined by the left ventricular end-diastolic pressure.
8.	Heart rate, which affects the duration of diastole, since coronary artery flow primarily occurs during diastole.

Factors that influence energy supply and energy demand in the myocardium were showed in Fig. (**1**).

Any clinical setting that reduces myocardial oxygen supply can cause ischemia and angina. The most frequent cause is coronary atherosclerosis, but other etiologies include coronary artery vasospasm, fibrosis, and embolism. In addition, stimulation of the esophagus by acid can cause coronary artery vasoconstriction and a reduction in coronary blood flow *via* a neural cardioesophageal reflex [8].

Angina can also occur after eating. Postprandial angina results from a redistribution of blood flow, away from territories supplied by severely stenosed coronary arteries to those supplied by less diseased or normal arteries [9]. This may be due to sympathetic activation from food ingestion, with norepinephrine-induced vasoconstriction in diseased vessels.

Fig. (**2**) illustrates that an imbalance between oxygen supply and demand can result from a diminished supply in the face of normal demands or increased demands that outstrip the blood supply. Primary, or variant, angina represents 'primary' decrease in the energy supply usually the result of a vasospastic problem. Secondary, or typical, angina represents a 'secondary' increase in myocardial oxygen demands exceeding the blood supply, thus resulting in myocardial ischemia [3].

PHARMACOLOGICAL TREATMENT OF STABLE ANGINA

The goals of pharmacological treatment of stable angina pectoris are to improve quality of life by reducing the severity and/or frequency of symptoms and to improve the prognosis of the patient. Pharmacotherapy is a viable alternative to invasive strategies for the treatment of most patients with stable angina pectoris and was actually associated with fewer complications than surgery or percutaneous coronary intervention (PCI) during a 1-year follow-up of MASS-II study [10]. An invasive treatment strategy may be reserved for patients at high risk or patients with symptoms that are poorly controlled by medical treatment.

The strategy for pharmacological relief of angina is based on improvement of the balance between myocardial oxygen supply and demand. For typical exertional angina (stable angina), this necessitates increasing the blood flow to heart or decreasing its work load. Treatment of variant angina is directed at reduction of vasospasm of the coronary vessels. Thus, symptoms of angina pectoris and signs of ischemia

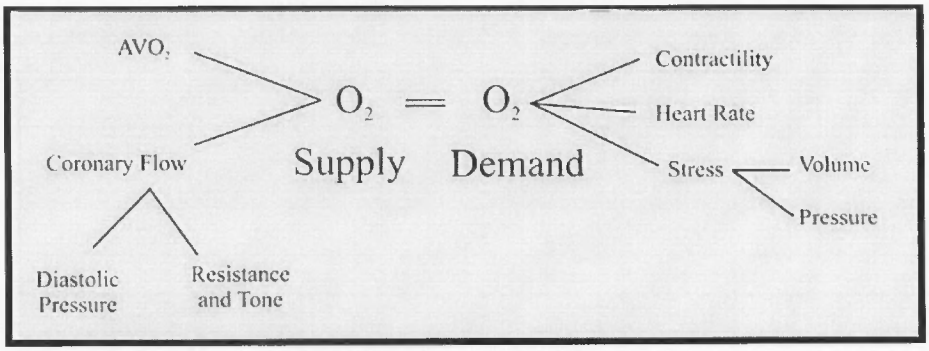

Fig. (1). Physiology of coronary circulation.
Legend: AVO_2 = arteriovenous oxygen content difference.

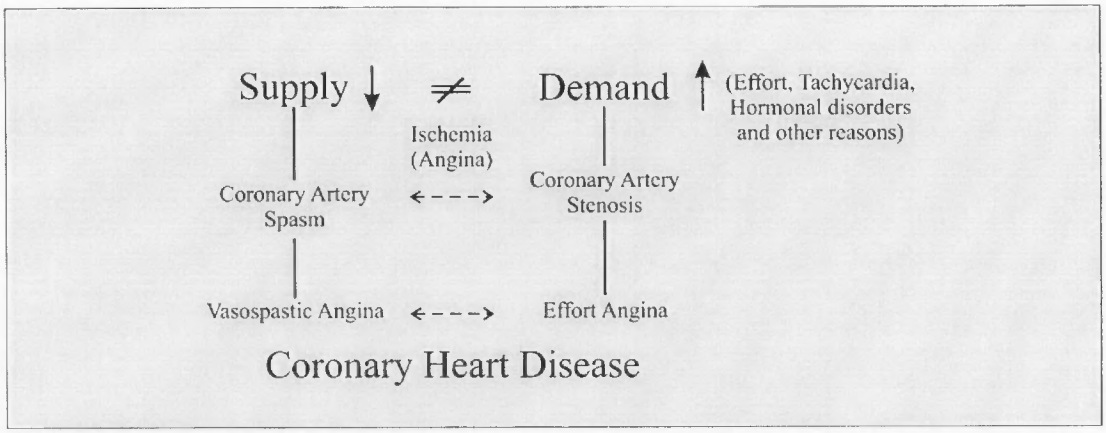

Fig. (2). Pathophysiology of coronary circulation.

(also silent ischemia) may be reduced by drugs that reduce myocardial oxygen demand and/or increase blood flow to the ischemic area. Commonly used antianginal drugs are beta-blockers, calcium antagonists, and organic nitrates [1,2].

Invasive and noninvasive methods of treatment in patients with stable angina pectoris were shown in Fig. (**3**).

This paper deals with some problems of organic nitrates. They have been for many years the cornerstone of antianginal therapy. However, nitrates have been also a source of numerous controversies related to their real efficacy and the mode of administration. The major controversies are connected with the occurrence of tolerance, and cross-tolerance which means that their clinical efficacy is decreased during long-term use.

ORGANIC NITRATES

Organic nitrates have been used in the treatment of angina pectoris for over one hundred years. NTG was first synthesized in 1846 by Ascanio Sobrero [11], who observed that a small quantity of the oily substance placed on the tongue elicited a severe headache. Constantin Hering, in 1847, developed the sublingual dosage form for NTG, which he advocated for a number of diseases [12,13]. The eminent English physician T. Lauder Brunton was unable to relieve severe recurrent anginal pain except when he bled his patient, and he believed that phlebotomy provided relief by lowering arterial blood pressure. The concept that reduced cardiac afterload and work are beneficial continues to present day. In 1857, Brunton administered amyl nitrite, a known vasodepressor, by inhalation, and he noted that anginal pain was relieved within 30 to 60 seconds [14]. The action of amyl nitrite was transitory, however, and the dosage was difficult to adjust.

NTG was used by Alfred Nobel to manufacture dynamite. It was in Nobel's dynamite factories in the late 1860s that the antianginal effect of NTG was discovered. Two interesting observations were made. First, factory workers on Monday mornings often complained of headaches that disappeared over the weekends. Second, factory workers suffering from angina pectoris or heart failure often experienced relief from chest pain during the work week, but which recurred on weekends. Both effects were attributed to the vasodilator action of NTG, which quickly became apparent to the physicians and physiologists in local communities.

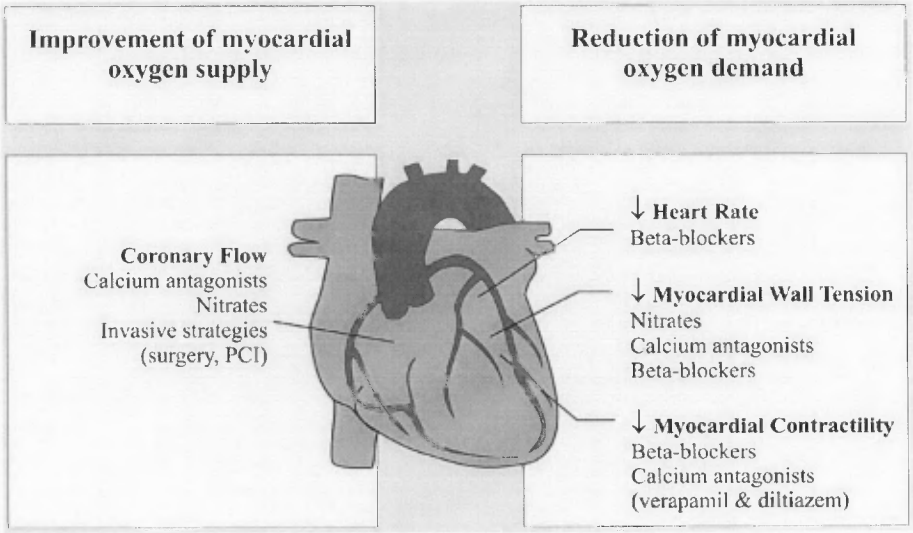

Fig. (3). Invasive and noninvasive methods of treatment in patients with stable angina pectoris.

Legend: PCI = percutaneous coronary intervention.

In 1879, William Murrell decided that the action of NTG mimicked that of amyl nitrite, he established the use of sublingual NTG for relief of the acute anginal attack and as a prophylactic agent to be taken prior to exertion [15]. Recently its antianginal efficacy following sublingual administration has been well established [16].

The other organic nitrates such as isosorbide dinitrate (ISDN), erythrityl tetranitrate, pentaerythritol tetranitrate (PETN) and manitol hexanitrate were developed and introduced into clinical practice when it was realized that the effect of sublingual NTG is short-lasting and that the effect of oral NTG is limited and unpredictable. ISDN, developed by Krantz in the first part of 20th century [17], has been used, like NTG, to relieve anginal symptoms. This agent can be administered by various routes, and in oral administration it has been confirmed as an effective antianginal drug [18]. Later, when the pharmacokinetic profile of the various nitrates was better understood and the therapeutic requirements in different cardiovascular diseases were better defined, new agents, such mononitrates, were developed and studied.

The use of organic nitrates in prophylactic clinical practice has largely been delayed by a conclusion made by group of investigators and reported first in 1972 and repeated thereafter. Needleman and co-workers [19] reported that orally administered organic nitrates were ineffective because of complete first-pass hepatic metabolism. Based on this thesis the use of long-acting organic nitrates declined markedly. It took almost a decade until the pharmacokinetic and pharmacodynamic profiles of ISDN and its metabolites (mononitrates) were fully understood. Only then was it possible for Needleman's thesis to be rejected on a strong scientific basis [20].

Isosorbide-5-mononitrate (IS-5-MN) is one of two major metabolites of ISDN, the other being isosorbide-2-mononitrate (IS-2-MN). It was initially pursued as a clinically useful agent as a result of suggestions that vascular effects of the parent drug were comparatively minor compared to those of these two metabolites. Subsequent work demonstrated that all three compounds are pharmacologically active and that elimination half-life of IS-5-MN is considerably longer than either the parent drug or the alternative metabolite. It has a number of advantages over ISDN including better absorption after oral administration and lack of first-pass hepatic metabolism. IS-5-MN was released in Germany in 1981 and has been widely used in clinical practice in Europe and in the other continents since than [21,22].

Nicorandil is an agent with a double cellular mechanism of action, acting both as a potassium channel activator and as a nitrate [2]. Therefore, it can be expected to cause less tolerance than nitrates do, and also cross-tolerance with classical nitrates does not seem to be a problem. In Japan, nicorandil is a standard antianginal drug, but it is not available in all countries.

The empirical observation that organic nitrates could be used safely for the rapid, dramatic alleviation of the symptoms of angina pectoris led to their widespread acceptance by the medical profession. However, in 1999 Nakamura *et al.* [23] have suggested that nitrate therapy may worsen the prognosis and survival in ischemic heart disease, although further studies are required to confirm these findings.

Recently nitrates, apart from beta-blockers and calcium antagonists, are still the mainstays of antianginal drug therapy. According to present European and American guidelines, all patients with angina should receive a prescription for sublingual NTG and instructions on its use. Long-acting nitrates should be considered for patients who cannot tolerate or fail to respond adequately to beta-blockers and calcium antagonists [1,2,24].

CHEMISTRY OF ORGANIC NITRATES

Organic nitrates are polyol esters of nitric acid, whereas organic nitrites are esters of nitrous acid. Nitrate esters (-C-O-NO_2) and nitrite esters (-C-O-NO) are characterized by a sequence of carbon-oxygen-nitrogen, whereas nitro compounds possess carbon-nitrogen bonds (C-NO_2). Thus, glyceryl trinitrate is not a 'nitro' compound, and it is erroneously called nitroglycerin (NTG). However, this nomenclature is both widespread and official.

Amyl nitrite is a highly volatile liquid that is administered by inhalation. Organic nitrates of low molecular weight (such as NTG) are moderately volatile, oily liquids, whereas the high-molecular-weight nitrate esters (e.g., erythrityl tetranitrate, PETN, ISDN) are solids. The fully nitrated polyols are lipid soluble, whereas their incompletely nitrated metabolites are soluble in water. In the pure form (without an inert carrier such as lactose), NTG is explosive. The organic nitrates and nitrites and several other compounds that are capable of conversion to nitric oxide (NO) have been collectively termed 'nitrovasodilators'. NO is thought to be the active intermediate in the action of this broad class of agents [25].

CARDIOVASCULAR EFFECTS

The action of organic nitrates is to relax vascular smooth muscle. Their vasodilator effects are evident in both systemic arteries (including coronary) and veins in normal subjects and in patients with ischemic heart disease, but they appear to be predominant in the venous circulation.

The venodilator effect reduces ventricular preload, which in turn reduces myocardial wall tension and O_2 requirements. The action of nitrates in reducing both preload and afterload makes them useful in the treatment of heart failure, as well as angina pectoris [26]. By reducing the heart's mechanical activity, volume, and O_2 consumption, in patients with exertional angina, nitrates improve exercise tolerance and extend the time to onset of angina and ischemia during exertion. When used in combination with calcium antagonists and/or beta-blockers, the antianginal effects appear greater.

Nitrates can reduce or reverse coronary vasospasm [27]. Thus, patients with primarily vasospastic angina or a large vasoconstrictor component to their angina can benefit from the direct coronary action of nitrate therapy.

Nitrates also indirectly improve subendocardial blood flow as the reduction in left ventricular end-diastolic pressure induced by systemic venous dilatation decreases the resistance to coronary blood flow from epicardium to endo-

cardium [28]. In addition, nitrates may lower the resistance to collateral vessel blood flow [29].

Nitrates have also an antithrombotic and antiplatelet effect. However, the clinical importance of these potentially beneficial effects is unclear [30]. Stimulation of platelet guanylate cyclase by nitrates prevents fibrinogen binding to platelet IIb/IIIa receptors, which is essential for platelet aggregation [31]. Transdermal nitroglycerin has been shown to inhibit platelet aggregation and thrombus formation in patients with angina [32].

MECHANISM OF ACTION

In the late 1970s and early 1980s, the vasodilator effect of NTG was discovered to be caused by NO, which was apparently generated from NTG in vascular smooth muscle [33-35]. These early observations on NO culminated less than 10 years later, in 1986, with the discovery that mammalian cells synthesize NO [36]. NO has been shown to be an extremely important signaling molecule in the cardiovascular system. In 1998, about 130 years after Alfred Nobel's invention of dynamite and the first observed clinical benefit of NTG, Furchgott, Ignarro and Murad were awarded the Nobel Prize in Medicine and Physiology "for their discoveries concerning nitric oxide as a signaling molecule in the cardiovascular system". Despite these achievements, the precise molecular mechanism by which NO is generated from nitroglycerin remained elusive until the work of Chen *et al.* [37].

Previous studies showed that the bioactivation of NTG somehow involved thiols or sulfhydryl-containing compounds, and that NO or NO-containing compounds constituted the biologically active species [33-36,38]. The earliest studies suggested that an interaction between nitroglycerin and sulfhydryl (-SH)-containing cellular receptors was necessary for vascular smooth muscle relaxation to occur and that repeated administration of nitroglycerin caused sulfhydryl depletion (*via* oxidation) and consequent tolerance to further vasodilation [38-40]. Subsequent studies addressing the activation of cytosolic guanylate cyclase by organic nitrate esters (nitroglycerin), organic nitrite esters (isoamyl nitrite), and nitroso compounds revealed that a chemical reaction occurred between the nitro compound and a thiol to generate an intermediate *S*-nitrosothiol, which then decomposed with the liberation of NO [35]. Tolerance to NTG was explained simply by thiol utilization and depletion in the presence of excess nitroglycerin, thereby resulting in deficient production of *S*-nitrosothiol and NO. This working hypothesis was supported by animal and clinical studies showing that the administration of relatively large doses of cysteine or *N*-acetylcysteine could prevent or reverse the tolerance to the vasodilator action of repeated administration of NTG. There were many unanswered questions associated with these earlier studies, however. The molecular mechanism of the interaction between nitroglycerin and thiol to generate *S*-nitrosothiol and NO remained an enigma. Moreover, the basis of the earlier hypotheses was activation of cytosolic guanylate cyclase in enzyme reaction mixtures and not vascular smooth muscle relaxation [35]. Isolated enzyme reaction mixtures or broken cell preparations are very different from intact cells or tissues. The early work with cellular extracts did not address the likely possibility that the reaction between nitroglycerin and thiol might be enzymatic in nature. In fact, the evidence was in favor of a nonenzymatic chemical reaction [35]. Subsequent studies suggested that one or more enzymatic mechanisms might be responsible for the bioactivation of NTG [41-47]. However, none of these enzyme systems could catalyze the selective formation of 1,2-glyceryl dinitrate from NTG and no correlation could be found between tolerance to NTG action and tolerance to enzyme activities. Chen *et al.* [37] uncovered the role of mitochondrial aldehyde dehydrogenase-2 (ALDH2 or mtALDH), which specifically generates 1,2-glyceryl dinitrate from NTG, in the bioactivation of NTG to elicit vasorelaxation and in the development of tolerance to NTG.

Chen *et al.* [37] used several ingenious approaches to elucidate the enzymatic mechanism of bioactivation of nitroglycerin: a source of large numbers of cells so that the lack of starting material would not be a limiting factor. By using mouse macrophages grown in cell culture, physiologically relevant, relatively low concentrations of nitroglycerin (0.1 μM) were shown to generate 1,2-glyceryl dinitrate through the catalytic action of an enzyme that was virtually identical to mouse mtALDH. ALDH2 purified from bovine liver showed identical catalytic properties to the mouse enzyme. Inhibitors of mtALDH, such as cyanamide and chloral hydrate, blocked the formation of 1,2-glyceryl dinitrate from nitroglycerin. ALDH2 possesses esterase activity [48] in addition to the classical NAD^+-dependent dehydrogenation activity, and the catalytic action on NTG was analogous to its esterase activity, with the important exception that nitrite (NO_2^-) rather than nitrate (NO_3^-) was a product of the enzymatic reaction. Thus, these observations were in agreement with the earliest biological findings that NTG is metabolized by tissues to inorganic nitrite or NO_2^- [35-36,38-40]. The classical sulfhydryl requirement for vascular smooth muscle relaxation by NTG [38] was explained as a chemical reaction between nitroglycerin and thiol sulfhydryl group to generate an intermediate *S*-nitrosothiol species, which then decomposed with the liberation of NO [35]. Other explanations and hypotheses were offered, but none of them could be replicated or confirmed across different tissues [49-50]. Therefore, the selective conversion of 1,2,3-glyceryl trinitrate (nitroglycerin) to 1,2-glyceryl dinitrate plus nitrite, together with the dependence on a reducing thiol cofactor, made mtALDH a compelling choice for the elusive enzyme pathway responsible for NTG bioactivation in vascular smooth muscle [51].

NITRATE TOLERANCE

The organic nitrates are effective antianginal drugs during initial treatment, but their therapeutic value is compromised by the rapid development of tolerance during sustained therapy. This means that repeated and prolonged administration of NTG and other organic nitrate esters causes the development of desensitization of vascular smooth muscle to further vasorelaxation by nitrates. This phenomenon has become a serious limitation to the chronic use of organic nitrate esters to treat angina pectoris. Thus, the potential for gradual attenuation of the pharmacological and therapeutic effects of organic nitrates during prolonged, and particularly continuous therapy, commonly designated 'nitrate tolerance'

induction, represents the Achilles heel of this group of drugs [52]. Numerous studies have shown that acute administration of various organic nitrates leads to impressive amelioration of myocardial ischaemia, and there is increasing evidence that they may play a pivotal role in the initial management of acute pulmonary oedema [53,54]. On the other hand, after prolonged administration of many, if not all, organic nitrates, beneficial effect is minimal [55].

MECHANISM OF NITRATE TOLERANCE

How nitrate tolerance occurs is incompletely understood. It is due to attenuation of the vascular effect of nitrates, not to altered pharmacokinetics [26]. Understanding the molecular mechanisms associated with the development of 'nitroglycerin tolerance' would undoubtedly lead to the discovery either of ways to avoid tolerance or of new NO-generating drugs that do not cause tolerance. At least several, not mutually exclusive, mechanisms have been proposed to explain the development of nitrate tolerance (Table **3**).

Table 3. Proposed Mechanisms of Nitrate Tolerance [37,56-64]

1.	Increased generation of vascular superoxide anion ($\cdot O_2^-$) [56,57]
2.	Plasma volume expansion [57]
3.	Impaired biotransformation of nitrates to NO [37,58]
4.	Decreased end-oxygen responsiveness to NO [59-61]
5.	Neurohormonal activation [57,62-64]

Accumulating data support the hypothesis that increased generation of vascular superoxide anion ($\cdot O_2^-$) is central to the process of nitrate tolerance [56,57]. There are multiple possible contributors to generation of oxygen free radicals, including effects of NTG on endothelial nitric oxide synthase (NOS) and counterregulatory neurohormonal activation. The consequences of increased superoxide anion formation are also multiple and include plausible links to many of the proposed mechanisms of nitrate tolerance.

Several studies reported that nitrate therapy causes plasma volume expansion. This observation led to the hypothesis that increased circulating volume and, subsequently, filing pressures, would counteract the NTG-induced decrease in preload, thus causing nitrate tolerance [57].

Impaired biotransformation of nitrates to NO is nitrate-specific and is not seen with non-nitrate sources of NO such as nitroprusside [58]. Consistent with this theory are the experimental observations that there is no tolerance to the effect of S-nitrosothiols and that the activity of mtALDH, the enzyme required for metabolism of nitrates to 1,2-glyceryl dinitrate in markedly reduced [37]. The same findings can be induced by inhibitors of mtALDH [37]. Chen *et al.* [37] demonstrated that in vascular tissue made tolerant to the vasorelaxant effect of NTG, a comparable decrease occurs in both mtALDH activity and tissue cyclic guanosine monophosphate (cGMP) accumulation [37]. Consistent with this observation is the report that mtALDH activity is markedly inhibited in patients undergoing chronic administration of NTG and other organic nitrate esters [50]. These findings also are consistent with previous reports that NTG tolerance in patients can sometimes be overcome by administration of *N*-acetylcysteine [36,58]. Chen *et al.* [37] showed that mtALDH is at least partially responsible for the bioactivation of NTG and is likely to be the target of nitroglycerin tolerance. Moreover, by understanding the molecular mechanism of nitroglycerin bioactivation and tolerance, it may now be possible to design and develop novel nitrovasodilator drugs that do not cause tolerance. One approach might be to develop drugs that do not engage mtALDH for the generation of NO. Ideally, the most appropriate kind of NO-donor drug might be one that is targeted to an enzyme that is selectively distributed to the vascular smooth muscle and acts as a substrate with only limited capacity to inhibit catalytic activity. Such a drug would be an effective vasodilator that could be used in combination with other drugs for the symptomatic treatment of hypertension. To be useful for the symptomatic treatment of angina pectoris, however, the drug would need to be targeted more to venous than arterial smooth muscle. Despite the desire to avoid tolerance, it may be a difficult task, indeed, to come up with an overall better antianginal drug than the 130-year-old NTG [51]. A relatively recent development is the suggestion that organic nitrates vary considerably in their potential to induce the development of tolerance. In particular, it has been suggested that PETN and possibly nicorandil may induce minimal release of superoxide anion, and hence minimal tolerance [52].

Consistent with a theory of decreased end-oxygen responsiveness to NO [59] is the finding in an animal study that vascular and hemodynamic tolerance to nitrates occurred despite high levels of NO and rates of NO formation that were similar in those animals that were not tolerant [60]. Also in support of this hypothesis is that transgenic animals that overexpress NOS have chronically elevated NO release, which is associated with reduced vascular reactivity to NO-mediated vasodilators [61].

Neurohormonal activation means an activation of the vasoconstrictor renin-angiotensin-aldosterone and sympathetic nervous systems in response to nitrate-induced vasodilation [62,63]. There is also increased peripheral sensitivity to these vasoconstrictors, an effect that can be reversed by angiotensin converting enzyme inhibition [62]. Abnormal coronary vasoconstrictor responses have also been described with continuous nitrate exposure [64].

A secondary implication of these emerging findings is that extended treatment with organic nitrates may have unfavorable consequences (free radical generation, endothelial dysfunction, and sympathetic activation) that could adversely affect long-term clinical outcomes. Such data raise a cautionary note that warrants additional investigation.

PREVENTION OF NITRATE TOLERANCE

The major drawback of long-term nitrate therapy is the development of tolerance. However, nitrate sensitivity in patients can be restored daily after a nitrate-free period of 8-12 hours [65,66]. Therefore, the only widely accepted method of preventing tolerance is the use of intermittent administration, independently of the type of preparation or route of administration [67-72].

Authors most frequently state that nitrate administration should be at 8 to 12 hour intervals, without specifying to what extent the length of interval is dependent on the type of nitrate and its pharmaceutical form and its dose [67-72]. However, in one study [73] it was showed that an almost linear dependence can be found between the duration of nitrate-free interval and the level of dose in oral administration of ISDN in sustained-release form. It should be 12 hr. nitrate-free interval for ISDN 40 mg; 18 hr. for ISDN 80 mg and 24 hr. for ISDN 120 mg. There are, however, two concerns regarding intermittent therapy:

1. A time-zero effect, which refers to a deterioration in exercise performance relative to placebo prior to the morning dose of nitrates.
2. Rebound angina, which refers to an increase in angina during the nitrate-free interval. There may result from a supersensitivity of the vessel wall to vasoconstrictors [74] or an increased vasomotor response to acetylcholine, suggesting the development of endothelial dysfunction [75].

Whether these effects occur to a clinically significant degree remains unclear [71]. Several other methods have been proposed to reduce nitrate tolerance, although none is as yet used clinically. Among them it could be mentioned, that co administration of nitrates with other vasodilators, such as captopril and hydralazine, may avoid the development of nitrate tolerance in patients with congestive heart failure [72].

CROSS-TOLERANCE

Nitrate tolerance is still an important limiting factor in the treatment of coronary heart disease. An additional clinical problem, which has not been fully resolved, is whether the development of tolerance to one organic nitrate in sustained therapy is connected with significant attenuation of the acute anti-ischemic effects of sublingual NTG. This is particularly relevant, because nitrate tolerance not only causes a decrease in the effectiveness of nitrates in the prevention of chest pain, but it may also restrict the effects of sublingual nitrates during anginal attacks. While nitrate tolerance is a widely known and treatable phenomenon, cross-tolerance, which can develop during concomitant therapy with two nitrate formulas, is less frequently acknowledged [52,66,76].

A recent development is the suggestion that cross-tolerance exists and could be clinically important [76]. Initial exposure to either ISDN or NTG led to marked prolongation of time to development of ischemic ECG changes; furthermore the acute effects of these agents were additive. Chronic continuous exposure to ISDN led to abolition of anti-ischemic effects, with simultaneous loss of effects of sublingual NTG. This demonstration cross-tolerance between ISDN and NTG is clinical evidence for the potentially damaging implications of this problem [76].

DIVERSITY OF ORGANIC NITRATES WITH RESPECT TO INDUCTION OF CLINICAL TOLERANCE

All long-acting nitrates seem to be equally effective, but the duration of antianginal effects of PETN in lower doses are not known. However, in higher doses (80 mg and 100 mg) PETN is an effective antianginal drug. A relatively recent development is the suggestion that organic nitrates vary considerably in their potential to induce the development of tolerance.

The tetranitrate PETN was previously shown to cause no induction of clinical tolerance and vascular oxidative stress [77-79]. The beneficial properties of PETN were explained by induction of the antioxidative proteins ferritin and heme oxygenase-1 (HO-1) [80,81] which may prevent oxidative stress and protect the vasculature from oxidative damage [82] and thereby mimic the antioxidant principle of compounds such as hydralazine [83]. HO-1 has been demonstrated to be a major protective and antioxidative principle in numerous therapeutic interventions [84,85]. The underlying mechanism of this protection is thought to be based on the breakdown of porphyrins to yield the potent antioxidant bilirubin [86-88] and the anti-inflammatory compound carbon monoxide [89,90] which is a weak activator of soluble guanylyl cyclase [91]. This is of special interest since HO-1 was reported to be localized within mitochondria [92] and thereby could directly affect nitrate-induced mitochondrial ROS formation and protect ALDH2 from oxidative inactivation.

There is growing body of evidence that ALDH2 only bioactivates tri- and tetranitrates which show high potency in tension studies. During reduction of nitrates the dithiol groups at the active site of the enzyme form a disulfide bridge causing its inactivation. It is thought that NTG induces mitochondrial ROS formation which may contribute to oxidative inhibition of ALDH2 activity and depletion of reduced thiols [93] thereby disrupting the physiological catalytic cycle. According to numerous reports in the literature PETN displays potent antioxidative properties which are probably based on the afore mentioned formation of bilirubin, CO and ferritin. Recently Mollnau *et al.* [94] demonstrated that bilirubin efficiently decreases mitochondrial oxidative stress in response to NTG treatment. Moreover, they could reproduce previous results of Oberle *et al.* [80] on PETN-triggered HO-1 induction and more importantly demonstrate that this inducing effect is not shared by NTG. This, however, could provide an explanation for the differences in tolerance induction by both organic nitrates.

One would expect that all NO donors induce HO-1 since this gene is heavily regulated by NO. However, previous publications have demonstrated that HO-1 inducing capacity is not shared by all NO donors [95]. It is unclear why ISDN and NTG do not induce HO-1. However, this could explain the different properties of ISDN and NTG versus PETN.

If the suggestion that PETN and possibly nicorandil may induce minimal tolerance is true, one would expect that prolonged exposure to PETN or nicorandil would not lead to diminution of responses to other organic nitrates such as NTG (that is, there would be no 'cross-tolerance'). However, further studies are needed to confirm this suggestion.

CONCLUSIONS

A major problem with the use of nitrates is the development of nitrate tolerance, which has been demonstrated with

all forms of nitrate administration delivering continuous, relatively stable blood levels of the drug. Several mechanisms of nitrate tolerance have been proposed. One of them is the hypothesis that increased generation of vascular superoxide anion is central to the process. During long-lasting nitrate therapy (except PETN), one can observe the development of reactive oxygen species (ROS) inside the muscular cell of a vessel wall, and these bind with nitric oxide (NO). This leads to decreased NO activity, thus, nitrate tolerance. PETN and possibly nicorandil has no tendency to form ROS, and therefore nitrate tolerance is absent or minimal. The only practical strategy to manage nitrate tolerance is to prevent it by providing a 'nitrate-free' interval. The optimal interval should be not shorter than 12-hour. A common form of nitrate withdrawal (rebound) is observed in patients whose angina is intensified after discontinuation of large doses of long-acting nitrates. Concomitant administration of other anti-anginal drugs could be resolution of the problem. In patients with stable angina treated with high doses of oral nitrates in long-term therapy, sublingual NTG maintains its full anti-ischemic effect both after initial oral ingestion and after intermittent long-term oral administration. However, sublingual NTG attenuates this effect during continuous treatment, when tolerance to oral nitrates occurs, and this is called cross-tolerance between sublingual and long-lasting nitrates. One would expect that prolonged exposure to PETN does not lead to diminution of responses to other organic nitrates such as sublingual NTG. Thus, during long-term PETN therapy, there is probably no tolerance or cross-tolerance, as during treatment with other organic nitrates. Long-acting nitrates are considered third-line therapy because a nitrate-free interval is required to avoid the development of tolerance. Therefore, nitrates should be considered for patients who cannot tolerate or fail to respond adequately to beta-blockers and calcium antagonists.

ABBREVIATIONS AND ACRONYMS

ALDH2 or mtALDH	=	Mitochondrial aldehyde dehydrogenase-2
AVO_2	=	Arteriovenous oxygen content difference
cGMP	=	Cyclic guanosine monophosphate
ECG	=	Electrocardiogram
HO-1	=	Heme oxygenase-1
ISDN	=	Isosorbide dinitrate
IS-2-MN	=	Isosorbide-2-mononitrate
IS-5-MN	=	Isosorbide-5-mononitrate
MASS-II study	=	The medicine, angioplasty, or surgery study
NO	=	Nitric oxide
NOS	=	Endothelial nitric oxide synthase
NTG	=	Nitroglycerin
$\cdot O_2^-$	=	Vascular superoxide anion
PCI	=	Percutaneous coronary intervention
PETN	=	Pentaerythritol tetranitrate
ROS	=	Reactive oxygen species

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Long-term creatine supplementation does not significantly affect clinical markers of health in athletes

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Abstract

Creatine has been reported to be an effective ergogenic aid for athletes. However, concerns have been raised regarding the long-term safety of creatine supplementation. This study examined the effects of long-term creatine supplementation on a 69-item panel of serum, whole blood, and urinary markers of clinical health status in athletes. Over a 21-month period, 98 Division IA college football players were administered in an open label manner creatine or non-creatine containing supplements following training sessions. Subjects who ingested creatine were administered 15.75 g/day of creatine monohydrate for 5 days and an average of 5 g/day thereafter in 5–10 g/day doses. Fasting blood and 24-h urine samples were collected at 0, 1, 1.5, 4, 6, 10, 12, 17, and 21 months of training. A comprehensive quantitative clinical chemistry panel was determined on serum and whole blood samples (metabolic markers, muscle and liver enzymes, electrolytes, lipid profiles, hematological markers, and lymphocytes). In addition, urine samples were quantitatively and qualitative analyzed to assess clinical status and renal function. At the end of the study, subjects were categorized into groups that did not take creatine (n = 44) and subjects who took creatine for 0–6 months (mean 4.4 ± 1.8 months, n = 12), 7–12 months (mean 9.3 ± 2.0 months, n = 25), and 12–21 months (mean 19.3 ± 2.4 months, n = 17). Baseline and the subjects' final blood and urine samples were analyzed by MANOVA and 2 × 2 repeated measures ANOVA univariate tests. MANOVA revealed no significant differences ($p = 0.51$) among groups in the 54-item panel of quantitative blood and urine markers assessed. Univariate analysis revealed no clinically significant interactions among groups in markers of clinical status. In addition, no apparent differences were observed among groups in the 15-item panel of qualitative urine markers. Results indicate that long-term creatine supplementation (up to 21-months) does not appear to adversely effect markers of health status in athletes undergoing intense training in comparison to athletes who do not take creatine. (Mol Cell Biochem **244**: 95–104, 2003)

Key words: ergogenic aids, nutrition, safety, exercise, renal function, muscle, metabolism

Introduction

Creatine is a naturally occurring amino acid that is obtained from the diet and/or synthesized endogenously from the amino acids glycine, arginine, and methionine [1, 2]. Approximately 95% of creatine is stored in skeletal muscle while the remaining 5% is stored the heart, brain, and testes [3]. Of this, approximately two thirds of creatine is stored as phos-

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phocreatine with the remaining creatine comprising the free creatine pool [4]. Creatine supplementation (e.g. 20 g/day × 5 days) has been consistently reported to increase muscle creatine and phosphocreatine typically by 15–40% [4–6]. Theoretically, increasing the availability of phosphocreatine would enhance cellular bioenergetics of the phosphagen system [1, 2, 5–7] as well as the shuttling of high-energy phosphates between the mitochondria and cytosol via the creatine phosphate shuttle [8–10].

In support of this contention, approximately 70% of studies that have evaluated the potential ergogenic value of creatine supplementation have reported significant improvements in performance particularly those involving high-intensity exercise and/or training [2, 3, 7]. There is also recent evidence that creatine supplementation may provide therapeutic benefit for patients with a variety of metabolic disorders [11–15], neuromuscular diseases [16–21], as well hasten recovery following immobilization [22]. These findings have indicated that creatine may serve as a promising ergogenic aid for athletes as well as may offer some clinical benefit for certain populations.

The only side effect that has been consistently reported has been weight gain which may be a desired effect for many athletes and patient populations [1–3, 7, 23]. Despite this apparent safety record, concerns have been raised in the popular media and scientific community regarding the safety of creatine supplementation [23–28]. In this regard, concerns have been raised that creatine supplementation may promote long-term suppression of creatine synthesis, increase renal stress, promote muscle and liver damage, alter fluid and electrolyte status, and/or cause unknown long-term side effects [26, 27]. In addition, creatine has been anecdotally suggested to increase gastrointestinal upset, cause diarrhea, promote cramping and dehydration, and increase the incidence of musculoskeletal injury [2, 23, 27].

Recent studies have attempted to determine the validity of these anecdotally reported concerns. For example, a number of studies have evaluated the effects of short-term creatine supplementation (e.g. 5 days–12 weeks) on thermal stress/dehydration [29–33], cramping [31, 34–43], electrolyte status [31, 40, 44–47], renal stress [48–55], muscle trauma [34–37, 39, 41, 43, 56, 57], and/or general markers of clinical health/safety [55, 58–65]. Several researchers have also attempted to retrospectively compare the medical status of self-reported creatine users (up to 5 years) to non-users [66, 67]. Other studies have attempted to use questionnaires to assess the prevalence of these potential side effects [38, 68–73]. However, most of these questionnaire based studies did not compare side effects of creatine users to non-creatine using controls and/or attempt to determine whether the side effects commonly reported in the media influenced responses to these questionnaires. Although results of these studies have consistently indicated that creatine supplementation does not appear to cause any of the anecdotally reported side effects, additional long-term research is warranted [1, 2, 26, 27]. The purpose of this study was to examine the short and long-term medical safety of creatine supplementation among athletes in comparison to athletes who did not take creatine during training and competition.

Materials and methods

Subjects

One hundred and sixteen National Collegiate Athletic Association (NCAA) Division IA college football players volunteered to participate in this study over a 2 year period. Subjects were informed as to the experimental procedures and signed informed consent statements in adherence with the Internal Review Board for use of human subjects in research at The University of Memphis and the American College of Sports Medicine. Of these, 98 subjects donated pre- and at least one subsequent blood and urine sample during the course of the study. Descriptively (means ± S.D.), subjects were 19.2 ± 2 years (range 18–23 years), 185 ± 8 cm (range 173–191 cm), 101 ± 18 kg (range 70–148 kg) and included a balanced representation of athletes from each position on the team (i.e. backs, receivers, tight ends, linebackers, lineman, and kickers).

Methods and procedures

Subjects were recruited to participate in this study during pre-season training prior to the 1998 and 1999 seasons. Approximately 65 subjects volunteered to participate during the first year and about 40 subjects volunteered to participate in the second year of the study. All subjects underwent pre-season medical examinations and were cleared to participate in football according to NCAA criteria. Subjects who volunteered to participate in the study chose whether they wanted to take creatine or non-creatine containing supplements during training. Subjects who chose to take creatine were administered in an open label manner 15.75 g/day of creatine monohydrate for 5 days and an average of 5 g/day thereafter in 5–10 g doses following supervised training sessions. Creatine was added to sports drinks or carbohydrate/protein drinks that were offered to players following training sessions, practices, and games by research assistants working with the strength and conditioning staff. Supplement intake was monitored and recorded in order to document creatine intake. When subjects were not on campus, they were provided creatine to take on their own and self-reported compliance. If for some reason a subject fell behind in taking creatine, subjects were administered up to 10 g/day in order to catch them up to an average of 5 g/day.

Fasting blood and 24-h urine samples were collected on as many athletes willing to provide samples at 0, 1, 1.5, 4, 6, 10, 12, 17, and 21 months of training (typically 30–55 per testing session). Collection of blood and urine samples coincided with the athletes reporting for summer school/preseason training (0 months), pre-fall football camp (1 month), post-fall football camp (1.5 months), post-season (4 months), start of spring semester/winter conditioning (6 months), and following spring football practice at the end of the spring semester (10 months) in the first year of supplementation. In the second year, blood and urine samples were obtained prior to fall football camp (12 months), at the end of the football season (17 months), and at of the end of the spring semester (21 months). Subjects who began the study in the second year donated blood and urine samples prior to the fall football season (0 months), after the football season (4 months), and/or at the end of the second semester (9 months).

Training consisted of summer resistance training/conditioning drills (1–2 h/day, 4 days/week), fall football camp (3–6 h/day, 6 days/week), practicing/competing during football season (2–4 h/day, 6 days/week) off-season resistance training/conditioning drills (1–2 h/day, 4 days/week), and spring football practice/resistance training (1–3 h/day, 4–5 days/week). Coaches, athletic trainers, and/or research assistants supervised all training sessions and games. Training duration, type, and general intensity as well as environmental conditions were recorded. Training averaged of 118 ± 68 min per session with an average intensity of 3.3 ± 1 on a 1–5 scale where 1 was equivalent to a walk-through practice prior to games and 5 was equivalent to game competition. Environmental conditions during training and competition ranged from 8–37°C (mean 24.2 ± 8°C) and 20–98% relative humidity (52.2 ± 16%). Injuries and medical conditions treated by athletic training/medical staff were recorded to assess medical status throughout the study. These data were reported in a companion paper presented at the 6th International Meeting on Guanidino Compounds in Biology and Medicine and published in the Journal of Molecular and Cellular Biochemistry [39].

Subjects observed an overnight 8-h fast prior to donating blood samples. Blood samples were obtained via venipuncture from an antecubital vein in the forearm using standard phlebotomy procedures between 6:00–9:00 am during each assessment period. Blood samples were collected into three 10 mL serum separation tubes (SST) and one 5 mL anticoagulant tube containing K3 (EDTA). The SST's were centrifuged at 5,000 rev/min for 10-min using a Biofuge 17R centrifuge (Heraeus Inc., Germany). Serum from two SST was transferred into microcentrifuge tubes and frozen at –80°C for subsequent analysis. Serum from the remaining SST was transferred into a 10 ml plain sterile tube. The plain and EDTA tubes were refrigerated and shipped overnight in cold containers to SmithKline Beecham Clinical Laboratories (Ann Arbor, MI, USA) for standard clinical analysis. A complete metabolic clinical chemistry panel was run on serum samples using the Olympus AU5200 automated chemistry analyzer (Melville, NY, USA) following standard clinical procedures. Cell blood counts with percent differentials and platelet determination were run on whole blood samples using a Coulter STKS automated analyzer using standard procedures (Coulter Inc., Hialeah, FL, USA). These analyzers were calibrated daily to controls according to manufacturers recommendations and federal guidelines for clinical diagnostic laboratories. Test to test reliability of performing these assays ranged from 2–8% for individual assays with an average variation of ±3%. Samples were run in duplicate to verify results if the observed values were outside control values and/or clinical norms according to standard clinical procedures.

Urine samples were collected in 24-h collection containers according to standard procedures. Urine volume was recorded and approximately 10 ml of urine was placed into a urine preservative tube, refrigerated, and shipped overnight in a cold container to SmithKline Beecham Clinical Laboratories to have a 15-item urinalysis performed using the Clinitek Atlas® automated urine chemistry analyzer (Bayer Diagnostics, Tarrytown, NY, USA). This analyzer was calibrated to controls according to manufacturers recommendations and federal guidelines for clinical diagnostic laboratories. In addition, approximately 10 ml of urine was pipetted into a plain sterile transfer tube and stored at –80°C for subsequent analysis. A microcentrifuge of frozen serum and a frozen urine transfer tube was shipped on dry ice to Nichols Institute (San Juan Capistrano, CA, USA) or to the Department of Biomedical Sciences at Queen's Medical Centre (Nottingham, UK) for determination of plasma creatinine, urine creatinine, and creatinine clearance using high performance liquid chromatography (HPLC) according to previously published procedures [6, 52, 53, 55].

Statistical analysis

At the end of the study, subjects were categorized as non-creatine users (n = 44); subjects who took creatine for 0–6 months (mean 4.4 ± 1.8 months, n = 12); subjects who took creatine for 7–12 months (mean 9.3 ± 2.0 months, n = 25); and subjects who took creatine for 12–21 months (mean 19.3 ± 2.4 months, n = 17). The subjects' baseline and final blood and urine samples were analyzed by MANOVA using SPSS for Windows Version 10.05 software (SPSS, Inc., Chicago, IL, USA). In addition, 2 × 2 repeated measures ANOVA univariate tests were performed on all dependent variables. Data were considered statistically significant when the probability of Type I error was 0.05 or less. Data are presented as means ± S.D.

Results

MANOVA revealed no significant differences ($p = 0.51$) between creatine users and non-users in the quantitative panel of blood and urine markers assessed. Since the clinical safety of creatine supplementation is of interest to the scientific and medical community, we have presented the means and standard deviations from the univariate repeated measures ANOVA tests performed on serum metabolic markers, muscle and liver enzymes, electrolytes, blood lipids, hematological markers, and quantitative urinary markers in Tables 1–6, respectively. No significant differences ($p > 0.05$) were observed among groups in any of the quantitative markers analyzed with the exception that significant interactions were observed in sodium, chloride, and hematocrit. However, as Tables 3 and 5 indicate, the differences observed among groups for sodium (141–142 meq/L), chloride (104–105 meq/L), and hematocrit (43–455%) were small, within normal ranges, and therefore do not appear to be of any physiological or clinical significance. No apparent differences were observed among groups in the qualitative urinary assessment of color, appearance, glucose, bilirubin, ketones, hemoglobin, total protein, nitrates, leukocyte esterase, white blood cells, red blood cells, epithelial cells, bacteria, amorphic cyrstals, or calcium oxalate.

Table 1. Metabolic markers for the creatine and non-creatine supplemented subjects. The pre-value is listed at the top and the post value is listed at the bottom of each cell

Variable	Non-creatine control (n = 44)	Creatine 0–6 M (n = 12)	Creatine 7–12 M (n = 25)	Creatine 12–21 M (n = 17)	Univariate interaction
Glucose	87 ± 11	90 ± 14	88 ± 15	84 ± 15	I = 0.16
(mg/dl)	85 ± 6	84 ± 12	84 ± 10	89 ± 9	
Total protein	7.3 ± 0.4	7.2 ± 0.7	7.3 ± 0.03	7.3 ± 0.4	I = 0.15
(g/dl)	7.3 ± 0.3	7.4 ± 0.3	7.2 ± 0.3	7.4 ± 0.4	
Albumin	4.4 ± 0.2	4.4 ± 0.3	4.4 ± 0.2	4.4 ± 0.2	I = 0.37
(g/dl)	4.4 ± 0.2	4.5 ± 0.1	4.4 ± 0.2	4.4 ± 0.2	
Globulin	2.9 ± 0.4	2.9 ± 0.3	2.9 ± 0.3	2.9 ± 0.2	I = 0.55
(g/dl)	2.9 ± 0.4	2.9 ± 0.3	2.8 ± 0.3	3.0 ± 0.3	
Albumin/globulin	1.55 ± 0.3	1.55 ± 0.2	1.56 ± 0.2	1.52 ± 0.2	I = 0.75
ratio	1.53 ± 0.2	1.56 ± 0.1	1.59 ± 0.2	1.48 ± 0.1	
Creatinine	1.23 ± 0.1	1.29 ± 0.2	1.26 ± 0.1	1.16 ± 0.2	I = 0.56
(mg/dl)	1.35 ± 0.1	1.41 ± 0.2	1.42 ± 0.2	1.35 ± 0.2	
Blood urea nitrogen	15.2 ± 3.8	15.2 ± 3.0	15.5 ± 3.8	15.6 ± 3.7	I = 0.85
(BUN) (mg/dl)	15.0 ± 2.9	15.9 ± 3.4	15.2 ± 2.6	15.6 ± 3.5	
BUN/creatinine	12.5 ± 3.3	12.0 ± 2.7	12.5 ± 3.5	13.8 ± 4.4	I = 0.52
ratio	11.2 ± 2.2	11.5 ± 3.1	10.7 ± 1.8	11.7 ± 3.2	
Uric acid	5.5 ± 1.1	5.5 ± 1.0	5.7 ± 1.5	5.3 ± 0.9	I = 0.78
(mg/dl)	5.4 ± 1.1	5.6 ± 1.5	5.5 ± 0.9	4.9 ± 1.0	

Data are means ± S.D.

Table 2. Muscle and liver enzymes for the creatine and non-creatine supplemented subjects. The pre-value is listed at the top and the post value is listed at the bottom of each cell

Variable	Non-creatine control (n = 44)	Creatine 0–6 M (n = 12)	Creatine 7–12 M (n = 25)	Creatine 12–21 M (n = 17)	Univariate interaction
Creatine kinase	796 ± 1124	427 ± 235	579 ± 373	862 ± 1821	I = 0.19
(U/l)	433 ± 146	469 ± 250	659 ± 545	355 ± 248	
Lactate dehydrogenase	180 ± 56	159 ± 91	159 ± 43	177 ± 63	I = 0.33
(U/l)	152 ± 21	151 ± 40	149 ± 38	143 ± 25	
Aspartate aminotransferase	34 ± 24	26 ± 11	28 ± 8	32 ± 22	I = 0.08
(U/l)	27 ± 4	31 ± 19	30 ± 8	25 ± 6	
Alanine aminotransferase	27 ± 11	22 ± 10	28 ± 11	27 ± 14	I = 0.45
(U/l)	25 ± 8	26 ± 13	26 ± 12		
Alkaline phosphatase	91 ± 29	91 ± 23	93 ± 25	101 ± 27	I = 0.09
(U/l)	93 ± 20	94 ± 31	100 ± 19	93 ± 17	

Data are means ± S.D.

Table 3. Serum electrolyte levels for the creatine and non-creatine supplemented subjects. The pre-value is listed at the top and the post value is listed at the bottom of each cell

Variable	Non-creatine control (n = 44)	Creatine 0–6 M (n = 12)	Creatine 7–12 M (n = 25)	Creatine 12–21 M (n = 17)	Univariate interaction
Sodium	141 ± 1	142 ± 3	142 ± 2	141 ± 2	I = 0.01
(meq/L)	141 ± 1	141 ± 1	141 ± 1	140 ± 1	
Chloride	104 ± 2	105 ± 3	105 ± 2	105 ± 2	I = 0.01
(meq/L)	103 ± 2	104 ± 2	103 ± 2	102 ± 2	
Potassium	4.5 ± 0.3	4.6 ± 0.5	4.7 ± 0.6	4.6 ± 0.3	I = 0.33
(meq/L)	4.4 ± 0.3	4.7 ± 0.7	4.5 ± 0.4	4.4 ± 0.2	
Phosphorus	4.3 ± 0.7	4.3 ± 0.6	3.9 ± 0.7	4.1 ± 0.5	I = 0.17
(meq/L)	4.5 ± 0.5	4.1 ± 0.4	4.3 ± 0.5	4.5 ± 0.8	

Data are means ± S.D.

Table 4. Blood lipid profiles for the creatine and non-creatine supplemented subjects. The pre-value is listed at the top and the post value is listed at the bottom of each cell

Variable	Non-creatine control (n = 44)	Creatine 0–6 M (n = 12)	Creatine 7–12 M (n = 25)	Creatine 12–21 M (n = 17)	Univariate interaction
Cholesterol	163 ± 29	174 ± 28	176 ± 27	164 ± 22	I= 0.29
(mg/dl)	172 ± 19	175 ± 22	179 ± 27	180 ± 26	
HDL	47 ± 10	46 ±7	45 ± 10	50 ± 13	I = 0.95
(mg/dl)	48 ± 13	47 ± 6	46 ±7	49 ± 11	
LDL	98 ± 24	111 ± 26	111 ± 22	98 ± 18	I = 0.35
(mg/dl)	106 ± 17	111 ± 22	111 ± 21	108 ± 25	
CHL/HDL	3.6 ± 1.1	3.8 ± 0.7	4.0 ± 0.9	3.5 ± 0.9	I= 0.46
ratio	3.8 ± 1.0	3.8 ± 0.6	4.0 ± 0.9	3.8 ± 0.8	
Triglycerides	92 ± 55	91 ± 40	97 ± 37	83 ± 34	I = 0.19
(mg/dl)	97 ± 44	86 ± 31	108 ± 56	110 ± 48	

Data are means ± S.D.

Discussion

Results of the present study indicate that short and long-term creatine supplementation (up to 21 months) does not appear to adversely effect clinical markers of health status in a large number of athletes undergoing intense training in comparison to athletes who do not take creatine. These findings provide the strongest evidence to date that long-term creatine supplementation does not appear to pose a health risk for athletes. In addition, these findings support previous reports from short-term studies (5 days–12 weeks) and long-term retrospective studies (up to 5 years) that creatine supplementation does not pose a health risk in apparently healthy individuals, athletes, or patient populations [38, 52, 53, 55, 58, 59, 64–66, 74]. The following discusses the results of the present study in consideration of concerns raised regarding the safety of creatine supplementation.

Concerns have been raised that creatine supplementation may increase renal stress and/or impair renal function. These concerns have been primarily fueled by reports of four case studies of possible renal dysfunction in individuals believed to have been taking creatine [75–78]. In each instance, elevations in serum creatinine (e.g. 1.5–1.7 mg/dl) were initially used to diagnose renal stress. Although the conclusions drawn about these case studies have been criticized [63, 79, 80] because these individuals had pre-existing kidney disease [77], may have been misdiagnosed [75], and/or apparently one subject ingested liquid creatine with only 25 mg of creatine per serving indicating that creatine could not have been related to the renal dysfunction observed [78], they have nevertheless raised concerns regarding the long-term safety of creatine supplementation.

Several studies have reported that creatine supplementation during training may increase serum creatinine levels (e.g. from 1.1 to 1.3–1.5 mg/dl). However, since creatine is naturally degraded to creatinine, the increased serum creatinine has been suggested to be due to a greater turnover of creatine following creatine loading and/or due to an ability to maintain a greater training volume/intensity following creatine supplementation [2, 44, 63]. Several recent studies have evaluated the effects of creatine supplementation on renal function by assessing urinary creatinine clearance [31, 48, 51–54] and/or using iohexol infusion techniques to assess glomular filtration [51]. These studies found that creatine

Table 5. Hematological markers for the creatine and non-creatine supplemented subjects. The pre-value is listed at the top and the post value is listed at the bottom of each cell

Variable	Non-creatine control (n = 44)	Creatine 0–6 M (n = 12)	Creatine 7–12 M (n = 25)	Creatine 12–21 M (n = 17)	Univariate interaction
White blood cells	5.5 ± 1.4	5.2 ± 1.0	6.0 ± 1.3	5.4 ± 1.3	I = 0.38
(thous/cum)	5.7 ± 1.0	5.0 ± 0.8	6.0 ± 1.2	5.9 ± 1 1	
Red blood cells	5.0 ± 0.3	4.9 ± 0.2	5.1 ± 0.4	5.0 ± 0.3	I = 0.28
(mil/cum)	5.1 ± 0.3	5.1 ± 0.3	5.1 ± 0.3	5.1 ± 0.3	
Hemoglobin	15.0 ± 1.1	15.0 ± 0.7	15.1 ± 1.0	14.6 ± 0.5	I = 0.22
(g/dl)	15.1 ± 0.9	15.3 ± 0.9	15.3 ± 0.7	15.2 ± 0.5	
Hematocrit	45 ± 3	43 ± 3	45 ± 3	44 ± 2	I = 0.03
(%)	45 ± 2	45 ± 2	46 ± 2	45 ± 1	
Total bilirubin	0.21 ± 0.16	0.22 ± 0.12	0.19 ± 0.01	0.22 ± 0.13	I = 0.22
(mg/dl)	0.16 ± 0.01	0.22 ± 0.01	0.13 ± 0.004	0.14 ± 0.007	
Mean corpuscle	90 ± 5	89 ± 5	88 ± 5	88 ± 4	I = 0.34
volume (fl)	89 ± 4	90 ± 3	89 ± 4	87 ± 4	
Mean corpuscle	30.0 ± 2	30.3 ± 1	29.7 ± 2	29.4 ± 1	I = 0.87
hemoglobin (pg)	30.2 ± 1	30.7 ± 1	29.9 ± 2	29.5 ± 1	
Mean corpuscle	30.6 ± 0.6	34.0 ± 0.6	33.5 ± 0.6	33.4 ± 0.6	I = 0.55
hemoglobin content (%)	33.7 ± 0.6	34.0 ± 0.4	33.5 ± 0.7	33.5 ± 0.7	
Red cell dimension	12.8 ± 0.7	12.3 ± 0.8	12.8 ± 0.8	12.8 ± 0.8	I = 0.86
width (%)	12.6 ± 0.6	12.2 ± 0.7	12.6 ± 0.5	12.6 ± 0.7	
Platelets	223 ± 38	205 ± 30	212 ± 48	226 ± 55	I = 0.89
(thous/cum)	227 ± 34	209 ± 33	219 ± 44	237 ± 51	
Mean platelet volume	9.8 ± 1.0	9.3 ± 1.1	9.9 ± 0.9	10.3 ± 2.9	I = 0.27
(fl)	9.5 ± 0.7	9.1 ± 1.1	10.0 ± 0.9	9.8 ± 1.6	
Neutrophils	47.7 ± 12	48.2 ± 10	51.7 ± 12	51.0 ± 11	I = 0.72
(%)	47.6 ± 10	49.6 ± 5	54.3 ± 9	49.4 ± 7	
Lymphocyges	38.7 ± 11	39.4 ± 11	35.5 ± 11	35.6 ± 9	I=0.36
(%)	39.6 ± 10	37.5 ± 6	33.2 ± 7	38.1 ± 6	
Monocytes	0.48 ± 0.2	0.40 ± 0.2	0.46 ± 0.2	0.44 ± 0.1	I = 0.91
(thous/cum)	0.47 ± 0.2	0.41 ± 0.2	0.44 ± 0.1	0.46 ± 0.1	
Eosonophils	0.24 ± 0.2	0.20 ± 0.1	0.26 ± 0.1	0.22 ± 0.2	I= 0.98
(thous/cum)	0.24 ± 0.1	0.20 ± 0.08	0.27 ± 0.2	0.22 ± 0.1	
Basophils	0.037 ± 0.04	0.040 ± 0.02	0.036 ± 0.03	0.036 ± 0.04	I = 0.59
(thous/cum)	0.034 ± 0.02	0.025 ± 0.02	0.032 ± 0.02	0.043 ± 0.04	
Neutrophil/	1.46 ± 0.9	1.39 ± 0.8	1.71 ± 1.0	1.57 ± 0.6	I = 0.69
lymphocyte ratio	1.32 ± 0.5	1.38 ± 0.4	1.80 ± 0.8	1.36 ± 0.6	

Data are means ± S.D.

Table 6. Quantitative urine markers for the creatine and non-creatine supplemented subjects. The pre-value is listed at the top and the post value is listed at the bottom of each cell

Variable	Non-creatine control (n = 44)	Creatine 0–6 M (n = 12)	Creatine 7–12 M (n = 25)	Creatine 12–21 M (n = 17)	Univariate interaction
Volume	1.50 ± 0.6	1.38 ± 0.8	1.67 ± 0.7	1.38 ± 0.5	I = 0.38
(l)	1.22 ± 0.3	1.05 ± 0.4	1.27 ± 0.6	1.37 ± 0.7	
Specific gravity	1.023 ± 0.006	1.024 ± 0.009	1.020 ± 0.006	1.019 ± 0.005	I = 0.24
	1.026 ± 0.004	1.024 ± 0.007	1.025 ± 0.005	1.025 ± 0.007	
pH	6.3 ± 0.6	6.1 ± 0.5	6.3 ± 0.6	6.1 ± 0.4	I = 0.36
	6.1 ± 0.3	6.2 ± 0.4	6.2 ± 0.4	6.1 ± 0.6	
Creatinine	2 .82 ± 1.6	2.45 ± 1.36	2.55 ± 1.2	2.67 ± 1.1	I = 0.76
(g/24 h)	2.30 ± 0.9	2.13 ± 0.8	2.47 ± 1.2	2.27 ± 1.8	
Creatinine clearance	269 ± 241	171 ± 117	234 ± 165	213 ± 150	I = 0.69
(ml/min)	162 ± 100	120 ± 63	168 ± 165	177 ± 185	

Data are means ± S.D.

supplementation has no apparent impact on renal function. Results of the present study support these findings in that no significant differences were observed among creatine and non-creatine users in serum creatinine, urinary creatinine excretion, or creatinine clearance.

Interestingly, baseline creatinine levels in the present study were at the upper end of normal for untrained individuals in all groups (i.e. 0.5–1.2 mg/dl) and the post-values were above normal values (1.35–1.42 mg/dl) in all groups. Although no significant differences were observed among creatine users and controls, most athletes had serum creatinine levels between 1.2–1.7 mg/dl particularly during more intense training periods. If one only used serum creatinine to diagnose renal stress, one could infer that many of these athletes were experiencing renal stress (regardless of whether they were taking creatine or not). Yet, no significant differences were observed among groups in creatinine clearance and values were within or exceeded norms (i.e. > 75–150 ml/min). It is also interesting to note that baseline creatinine clearance values (typically obtained prior to fall football season) were above norms (171–269 ml/min) and that post creatinine clearance values analysis (typically obtained at the end of the football season or at the end of the second academic semester) decreased in all groups from pre- to post analysis (suggesting a decreased renal function) but remained within or slightly above clinical norms (120–177 ml/min). These findings indicate that renal function may vary among college football players possibly due to their large body mass and/or the type and volume of training they are engaged. Consequently, it is our view that care must be taken when interpreting individual changes in serum creatinine and/or creatinine clearance in these types of athletes and inferring that creatine supplementation may have been related to changes in these renal markers when large variations in these variables are apparently normal for this population.

Concerns have also been raised that creatine supplementation may increase muscle and/or liver damage [2, 27, 55]. This concern has been based on an initial report suggested that athletes taking creatine during training may experience slightly elevated muscle and/or liver enzymes [44]. Although the levels reported were within normal values for athletes, some have suggested that creatine may increase muscle and/or liver damage. Results of the present study indicated that athletes engaged in intense training have creatine kinase (CK) levels above clinical norms for untrained individuals (i.e. > 225 IU/L). However, the mean values observed were within normal ranges for athletes engaged in intense training (i.e. typically 250–1,000 IU/L) and no significant differences were observed among creatine and non-creatine users in CK values. In addition, no significant differences were observed among creatine and non-creatine supplemented groups in lactate dehydrogenase (LDH), aspartate aminotransferase (AST), or alanine aminotransferase (ALT) values and all of these values were within normal ranges (i.e. LDH 100–250; AST and ALT < 55 IU/L) for non-athletes. These findings indicate that although these athletes may have had elevated CK levels, there does not appear to be any difference in muscle and liver enzyme efflux among athletes who do and do not take creatine during intense training and competition.

Creatine supplementation has also been suggested to alter fluid balance and/or electrolyte status. The basis of this concern was from initial reports suggesting that urine output may decrease slightly during creatine loading thereby suggesting that short-term creatine supplementation may increase fluid retention [6]. Although subsequent studies have been unable to demonstrate a disproportionate increase in total body water following creatine supplementation [31, 40, 44, 46], the potential increase in fluid retention has been theorized to dilute electrolytes and predispose athletes to cramping. Results of the present study do not support this hypothesis. Although significant interactions were observed among groups in sodium and chloride levels, differences among groups were negligible (i.e. < 1 meq/L) and of no physiological or clinical significance. In addition, no significant differences were observed among groups in potassium, calcium, phosphorus, urine output, or urine specific gravity. Moreover, as reported in our companion paper to this study, creatine supplementation did not increase the incidence of dehydration or muscle cramping in these athletes monitored over a 3-year period [39]. These findings support results of previous studies indicating that creatine supplementation does not increase thermal stress or promote dehydration [29–33, 46, 47, 81], cramping [31, 34–43], or alter electrolyte status [31, 40, 44, 45].

Earnest and colleagues [82] reported that creatine supplementation lowered blood lipids in a group of subjects with high triglycerides. Since then, several researchers have examined whether creatine supplementation affects blood lipid profiles [44, 83–85]. One theory for this phenomenon is that creatine supplementation may enhance the quality of training thereby accentuating the positive effects of exercise on blood lipid profiles. Potentially, a creatine induced reduction in cholesterol and/or triglyceride levels may have significant health benefits. Although we previously reported that blood lipid profiles were improved during the initial 42-days of this study as subjects went through pre-season training and football camp [86], no significant differences were observed among creatine and non-creatine users in our one-year analysis [62] or in the present overall analysis. These findings suggest that the possible influence of creatine on lipid profiles in athletes with normal blood lipids is either transient or non-existent. Present findings support other reports indicating creatine supplementation does not appear to affect blood lipid levels in athletes [55, 67, 84, 85]. However, additional research should examine the potential influence of creatine supplementation on lipid profiles particularly in individuals with elevated cholesterol and/or triglycerides.

The last major concern about creatine supplementation that we would like to address is that creatine supplementation may cause unknown long-term side effects. Results of the present study do not support these contentions. In this regard, we saw no evidence that short- or long-term creatine supplementation caused any clinically significant change in serum metabolic markers, muscle and liver enzyme efflux, serum electrolytes, blood lipid profiles, red and white whole blood cell hematology, or quantitative and qualitative urinary markers. These findings support other reports indicating that long-term creatine supplementation (up to 5 years) did not appear to cause clinically significant side effects in various patient populations and/or retrospective analysis of athletes [52, 55, 64–66]. Although research should continue to evaluate the health consequences of creatine supplementation (particularly in adolescents and various patient populations), results of this study indicate that creatine supplementation (~ 5 g/day for up to 21 months) appears to be a safe nutritional supplement for athletes engaged in intense training and competition.

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Intragastric formation and modulation of N-nitrosodimethylamine in a dynamic in vitro gastrointestinal model under human physiological conditions

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Abstract

Human exposure to carcinogenic N-alkylnitrosamines can occur exogenously via food consumption or endogenously by formation of these compounds through nitrosation of amine precursors. Information on the intragastric formation of NDMA from complex mixtures of precursors and inhibitors in humans is not available. In this study the formation of N-nitrosodimethylamine (NDMA) has been quantitatively analysed in a dynamic in vitro gastrointestinal model, in which gastric conditions can be modulated and closely simulates the physiological situation in humans. Substantial amounts of NDMA were produced when nitrite and dimethylamine or codfish were simultaneously introduced into the model. However, humans are gradually exposed to nitrite by the intake of nitrate-containing food. Nitrate secreted in saliva is converted to nitrite by oral bacteria. To mimic the human exposure to nitrite in a realistic way, nitrite was gradually added into the gastric compartment, simulating the swallowing of nitrite containing oral fluid after the intake of nitrate at the level of 0.1–10 times the ADI. Under these conditions, the cumulative amounts of NDMA formed were 2.3–422 μg NDMA and 1.8–42.7 μg NDMA at a rapid and slow gastric pH decrease, respectively. Beside codfish, various fish species and batches in combination with nitrite, simulating the intake of for times the ADI of nitrate, were investigated. Herring, pollack and plaice were also able to induce NDMA formation. Mackerel, salmon and pike perch did not result in increased NDMA formation. Furthermore, the effect of nitrosation modulators on NDMA formation was investigated. Thiocyanate (2 mM) increased NDMA formation, but the increase was not statistically significant. In contrast, orange jus and tea effectively, but not totally, reduced the amount of NDMA formed in the gastric compartment. These experiments show that (1) the dynamic in vitro gastrointestinal model is an appropriate tool for mechanistic studies on the intragastric formation of nitrosamines, and (2) that the results obtained with this model are helpful in evaluating human cancer risk for the combined intake of codfish-like fish species and nitrate-containing vegetables.

Keywords: Gastrointestinal model; N-nitrosodimethylamine; Nitrite; Codfish; Ascorbic acid; Toxicokinetic model

1. Introduction

N-alkylnitrosamines are potent carcinogens. They induce tumours in a variety of animal species, such as rat, mouse, rabbit, fish and birds and in different organs such as the bladder, kidney, liver, oesophagus or stomach (Peto et al., 1984; IARC, 1987). For the following reasons, it is assumed that N-alkylnitrosamines can induce tumours in humans as well. First because human exposure to N-alkylnitrosamines has been associated with increased risk for e.g. gastric, bladder and colon cancer (Bartsch et al., 1990; Knekt et al., 1999; Mirvish, 1995). Second because biotransformation and biological activity of these nitrosocompounds in animals appear to be similar to those in humans (Preussmann, 1990). The IARC evaluated various N-nitrosamines as Group 2A (probably carcinogenic to humans), such as N-nitrosodimethyl-amine and N-nitrosodiethylamine, and as Group 2B (possibly carcinogenic to humans), such as N-nitrosodiethanolamine (IARC, 1978, 2000). However, epidemiological studies on N-alkylnitrosamines

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and cancer risk have yielded inconsistent findings (reviewed by Eichholzer and Gutzwiller, 1998).

Human exposure to N-alkylnitrosamines can occur via two pathways, namely via exogenous and endogenous routes (extensively reviewed by Tricker, 1997; Walker, 1990; Shephard et al., 1987). Exogenous exposure may result from consumption of food or beverages (e.g. beer), inhalation of tobacco smoke, the use of rubber articles (e.g. teats and soothes) and cosmetics, or from occupational exposure in the rubber industry (Lijinsky, 1990; Tricker and Peussmann, 1991; Gray and Stachiw, 1987; Straif et al., 2000). Endogenous exposure results from the nitrosation of precursors of nitrosamines in the human body. The nitroso moiety is derived from nitrite, which reacts with ingested secondary amines to form N-nitrosamines under acidic conditions. These conditions prevail in the stomach (Mirvish, 1995; Leaf et al., 1989).

A minor part of nitrite exposure occurs via nitrite-containing food, such as preserved meat and certain vegetables, but the concentration is generally very low (Gangolli et al., 1994; MAFF, 1992). The main route of exposure is from the reduction of nitrate into nitrite by oral bacteria (Stephany and Schuller, 1980; Walters and Smith, 1981). Nitrate in food or drinking water is absorbed in the small intestine. Roughly 25% of the intake is actively secreted by the salivary glands into the oral cavity. Of this fraction approximately 20% (and thus 5% of dietary nitrate) is converted into nitrite by oral bacteria (Spiegelhalder et al., 1976). This nitrite enters gradually the stomach after swallowing of oral fluid. At low pH, nitrite will be converted to nitrous acid and subsequently to N_2O_3, which reacts with secondary amines in its deprotonated form (e.g. from food sources such as fish), to give nitrosamines (Mirvish, 1983). The optimum pH for the nitrosation reaction, as determined in in vitro studies, is between 2.0 and 3.4, depending on the type of amine (Mirvish, 1975). In addition, nitrosation has also been reported at neutral pH by nitrosating bacterial species (Mirvish, 1995; Leaf et al., 1989).

In order to assess the daily exposure to exogenous N-alkylnitrosamines, sensitive and precise gas chromatography (GC-) methods are currently available for the detection of N-alkyl-nitrosamines. In the Netherlands the dietary intake of the volatile N-nitrosodimethylamine (NDMA) is low, approximately 0.1 μg per person per day (Ellen et al., 1990). The accuracy of the quantification of endogenous formation of N-alkylnitrosamines in the human body and the contribution of endogenously formed N-alkylnitrosamines to the total exposure to N-nitrosamines is still a matter of debate (Gangolli et al., 1994). Matechematic models (Licht and Deen, 1988) and in vivo studies with N-nitrosoproline (NPRO), formed out of L-proline and nitrite, have indicated that the endogenous exposure is possibly much less important than exogenous exposure (Bartsch and Spiegelhalder, 1996). However, NPRO is a non-carcinogenic nitrosamine, it is not metabolized and excreted almost completely in the urine (Ohshima and Bartsch, 1981; Shapiro et al., 1991). It is not clear how to extrapolate this finding to the intragastric formation of carcinogenic N-nitrosamines.

Vermeer and colleagues demonstrated that the intake of nitrate at the level of the acceptable daily intake (ADI; 3.65 mg/kg bw) via drinking water or vegetables in combination with a meal containing different types of amine-rich fish, led to significantly increased urinary NDMA excretion (0.64–0.87 μg/24 h) by human volunteers (Vermeer et al., 1998; van Maanen et al., 1998). Spiegelhalder and Preussmann (1985) assumed that at least 0.5% of the NDMA is excreted in the urine, which means that substantial amounts of NDMA are produced in the stomach.

Although it is indicated that NDMA can be formed in the stomach, only a crude estimate can be given of the actual amount. It could be argued that a more accurate estimate might be obtained by in vivo measurement of NDMA formation. However, for practical and ethical reasons a study of NDMA formation in the stomach of human volunteers is very difficult. We therefore used an alternative way to estimate the formation of NDMA in the human stomach, i.e. with a dynamic in vitro gastrointestinal model, consisting of four compartments that represent the stomach, duodenum, jejunum and ileum (Minekus et al., 1995). The model closely simulates the human physiological situation after the intake of food. In a previous publication, it has been demonstrated that this gastrointestinal model is a useful tool to study the availability and interaction of food mutagens (e.g. heterocyclic amines) and antimutagens, such as black and green tea extracts (Krul et al., 2000).

In the gastrointestinal model the nitrosation reaction was investigated between nitrite and dimethylamine (DMA) and codfish under different gastric pH conditions: a slow and a more rapid pH decrease. Nitrosation of DMA was studied because DMA is often present at high concentrations in food (e.g. codfish) and the nitrosation product NDMA is one of the most potent carcinogenic N-alkylnitrosamines in rodents.

To simulate realistically the swallowing of nitrite-containing oral fluid that has been formed from nitrate by the microflora in the oral cavity, the formation of nitrite was quantified with the aid of a toxicokinetic model. With this model the flow of nitrite-containing oral fluid into the stomach was calculated for the intake of different levels of nitrate (0.1–10 times ADI) and incorporated in the gastrointestinal model. Beside codfish, we determined possible differences in NDMA formation for a variety of frequently consumed fish species, such as herring, mackerel, plaice, pollack and salmon.

Thiocyanate catalyzes the formation of nitrosamines, especially under acidic conditions (Boyland and Walker,

1974). Thiocyanate might be secreted by salivary glands or directly into the gastric juice (Dougall et al., 1995). It has been established that endogenous NOC formation occurs at a higher level in smokers (Hoffmann and Brunneman, 1983). An explanation might be the higher level of thiocyanate present in saliva of smokers (5.5±0.2 mM) compared to non-smokers (1.8±0.2 mM) (Walters et al., 1979). We investigated the influence of thiocyanate (2 mM) on the formation of NOC after the consumption of codfish, spinach and nitrite at the level of two times the acceptable daily intake (ADI) of nitrate.

On the other hand various antioxidants, such as ascorbic acid and polyphenols, showed inhibitory effects on the formation of nitrosamines (Bartsch, 1988). The intragastric ascorbic acid concentration is determined by consumption of fresh fruit and vegetables, and by active secretion of ascorbic acid by the gastric mucosa. Ascorbic acid inhibits NOC formation by a rapid reduction of nitrosating species, such as N_2O_3 into nitric oxide (NO). The ability of ascorbic acid to remove nitrite is however markedly reduced by the presence of oxygen, because NO can then react with oxygen to reform nitrite.

Experiments with respect to the inhibition of NDMA formation were performed with two inhibitors, *viz.* orange juice (as a source of ascorbic acid) and black tea (which contains large amounts of polyphenols).

2. Materials and methods

2.1. Chemicals and food samples

The digestive juices and enzymes used in the in vitro digestion model were lipase, (150 U/mg; *Rhizopus* lipases F-AP 15, Amano Pharmaceuticals) and pepsine (2100 U/mg; Sigma, Zwijndrecht, the Netherlands). N-nitrosodiisopropylamine (NDiPA) in methanol was supplied by Schmidt (Prochem). Nitrosodimethylamine (NDMA), dimethylamine and sodium nitrite were purchased from Sigma (Zwijndrecht, the Netherlands). All other chemicals were purchased from Merck (Darmstadt, Germany). Freshly pasteurised codfish fillet from the North Sea was obtained from a Dutch fish supplier and stored at −20 °C.

Lyophilized green tea and black tea were kindly provided by Unilever Research Vlaardingen (the Netherlands). Deep-frozen, chopped spinach (Iglo, the Netherlands) was obtained from a local supermarket. The various species of frozen fish, codfish, salmon, pollack, plaice and pike perch were obtained from fish suppliers and stored at below −18 °C. Smoked mackerel, and salted herring were not frozen, but stored overnight at 4 °C.

2.2. The dynamic in vitro gastrointestinal model

The TNO in vitro gastrointestinal model is a dynamic, multi-compartmental, computer-controlled system that mimics the physiological processes in the human stomach and small intestine (Minekus et al., 1995; Krul et al., 2000). In the model the body temperature, pH, peristaltic movements and secretion of digestive enzymes, are simulated. These parameters and variables can be handled in a controlled and standardised way.

Artificial gastric juice containing 3.1 g/l NaCl, 1.1 g/l KCl, 0.6 g/l $NaHCO_3$ and 0.15 g/l $CaCl_2.2H_2O$ is added into the model at a rate of 0.5 ml/min. Lipase (500 mg/l) and pepsin (150 mg/l) are separately added into the gastric compartment at a rate of 1.0 ml/min. The model is made of glass jackets with flexible walls inside, which are surrounded by water of 37 °C. The water pressure squeezes the walls; its relaxation and compression ensure mixing and simulate the peristaltic movements. Valves mimicking the pyloric sphincter control the transit of the gastric content to the duodenal compartment. For the experiments described, the gastric compartment of the model was used with some minor modifications (Fig. 1).

The pH in the gastric compartment of the model was continuously measured and accurately adjusted by the addition of 1 M HCl. This offered the opportunity to define every desirable pH curve, in combination with a programmed gastric emptying rate. In the first set of experiments two different physiological conditions were simulated. Namely, the condition after intake of a homogenised, low-caloric solid meal, with gastric pH set to decrease slowly and the condition after intake of a semi-liquid meal, in which the pH in the gastric compartment decreased more rapidly. The gastric emptying rate was the same in all experiments and simulated the gastric delivery of a low-caloric meal (Moore et al., 1984) (Fig. 2).

Three hours after the addition of food the emptying of the gastric compartment was complete for more than 95% (Fig. 2). During this period gastric delivery in 1h aliquots (0–1 h, 1–2 h, and 2–3 h) was collected on ice. The further reaction of the precursors nitrite and DMA to NDMA after collection of fractions from the gastric compartment was prevented by the immediate addition of 10M NaOH to reach a pH >10. The volumes of the 1 h fractions were measured and the samples were stored at <-70 °C until analysis of NDMA concentrations. Except in experiments described in Section 2.4.5, the fractions collected between 0 and 1 h and between 1 and 2 h were pooled. The pooled sample (0–2 h) and the 2–3 h fraction were analysed. The model was protected from UV light by UV-absorbing foil. All experiments were performed in duplicate, unless otherwise indicated.

2.3. Analysis of NDMA

NDMA was analysed in the gastric samples by gas chromatography in combination with a Thermal Energy Analyser detector (GC-TEA) as described by Pensabene, with some minor modifications (Pensabene and Fiddler, 1994; Dallinga et al., 2001). Quantification of the NDMA content was based on the peak areas of NDMA and the internal standard (N-nitrosodiisopropylamine) using linear regression. The detection limit of the method was approximately 80 pg injected, which would correspond to an NDMA concentration of 1.6 μg/l gastric juice. The cumulative amounts of NDMA formed, were calculated based on concentration in the samples and the volume of the delivered gastric content. For confirmation purposes a selection of extracts in which NDMA was detected, were irradiated for three hours with UV-light (254 nm). On subsequent GC-TEA re-analysis all peaks due to the presence of NDMA has disappeared. This means that the initial peak was indeed NDMA.

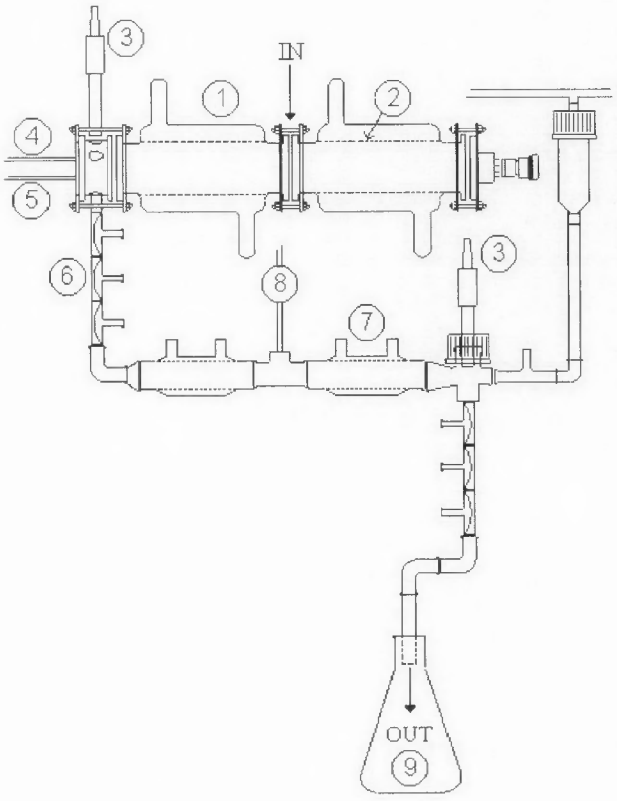

Fig. 1. Schematic view of the gastric model: (1) gastric compartment, made of glass jackets, (2) flexible wall, surrounded by 37 °C water, to ensure peristaltic movements, (3) continuous measurement of the pH and addition of HCl to follow the preset pH curve, (4) addition of lipase and pepsin, (5) addition of nitrite simulating the swallowing of oral fluid, (6) valve system that mimics the pyloric sphincter, (7) compartment for the termination of the nitrosation reaction, (8) addition of NaOH, 9) collection of the samples on ice.

2.4. Experiments

2.4.1. NDMA formation measured with DMA and a fixed dose of nitrite

To investigate whether the formation of NDMA can occur under the experimental conditions of the in vitro model, the gastric compartment was loaded with 300 ml of 0.1M sodium citrate buffer (pH 6.8) containing 5 mM DMA and 5 mM sodium nitrite. In control experiments the model was loaded with 0.1 M sodium citrate buffer (pH 6.8) or 5 mM DMA dissolved in 0.1 M sodium citrate buffer (pH 6.8), both without nitrite. These experiments were performed to verify that NDMA, possibly released from the rubber components of the gastric compartment or as contamination of the DMA solution, was not detected after passage through the model.

2.4.2. NDMA formation measured with codfish and a fixed dose of nitrite

Codfish was used to investigate whether amines such as DMA are released from a food matrix under the physiological gastrointestinal conditions as simulated in the in vitro model, and whether these amines can act as nitrosation precursors. The fish was pasteurised (20 min at 70 °C), cooled down to 10 °C within 1 h, and stored at −20 °C. Before use, 100 g of frozen codfish was thawed and thereafter heated in a microwave oven (55 s, 600 W) and then crushed and mixed with 5 mM sodium nitrite in 0.1 mM citrate buffer with a blender. The mixture was loaded into the gastric compartment. Endogenous NDMA was measured in a control experiment in which the model was loaded with codfish without nitrite.

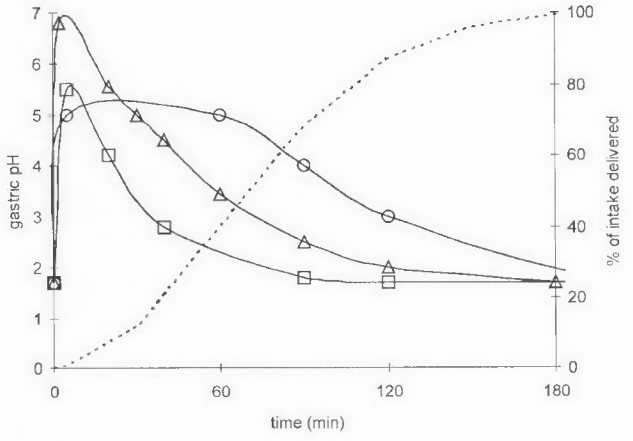

Fig. 2. The pre-set pH curves in the gastric compartment simulating a rapid (squares), moderate (triangles) and slow (circles) gastric pH decrease. The gastric delivery rate (dotted curve) was the same in all experiments.

2.4.3. NDMA formation measured with codfish and gradual addition of nitrite

To simulate the nitrosation of DMA under more physiological conditions, the nitrite solution was gradually added to the gastric compartment at a rate which mimics the swallowing of nitrite-containing oral fluid, after uptake of nitrate from food (0.1–10 times the ADI of nitrate). The rates were calculated with the aid of a toxicokinetic model, based on human data (see below). Experiments were performed with 5 mM DMA and nitrite secretion simulating the intake of nitrate at 10 times its acceptable daily intake (ADI, 3.65 mg nitrate/kg body weight). Subsequently, experiments were performed with 100 g of codfish and transport of nitrite corresponding to the intake of 0.1, 1, 5 and 10 times the ADI of nitrate at a slow and rapid gastric pH decrease (except five times the ADI of nitrate, which was only performed under the conditions of a slow gastric pH decrease). This range represents the human exposure to nitrate, the median adult exposure being 1 mg/kg/day with a range of 0.1–20 mg/kg/day (10 times ADI corresponds to 36 mg/kg/day) (Slob et al., 1995). DMA and codfish, respectively, were loaded into the model at once at the start of the experiment.

2.4.4. NDMA formation measured with various fish species and batches

Frequently consumed fish species (89% of the total variants) were selected based on the Dutch food consumption bearing (1997–1998), to investigate the NDMA formation. Five batches each of codfish and herring, four batches each of salmon, mackerel, plaice, and pollack and one batch of pike perch were analysed. Before use, 100 g of fish was thawed, heated in a microwave oven (3 min, 600 W) and thereafter crushed and mixed with artificial gastric juice. Salted herring and smoked mackerel were not heated, because generally they are consumed cold.

Nitrite was gradually added into the gastric compartment, simulating the swallowing of oral fluid containing nitrite after the intake of five times the acceptable daily intake of nitrate. Control experiments were performed in which either one of the precursors for NDMA formation was omitted: 0.1 M sodium citrate buffer pH 6.8 with nitrite only, and herring without the addition of nitrite. In addition, one experiment was performed with pike perch, which is supposed not to contain DMA. Nitrite was added simulating the intake of 10 times the ADI of nitrate.

2.4.5. Modulation of NDMA formation, influence of thiocyanate and antioxidants

First 70 g codfish (prepared as described above) was introduced into the gastric compartment. Thereafter, to simulate the nitrosation of amines in codfish under more physiological conditions defrosted spinach (50 g) was mixed with the homogenised codfish and introduced into the model.

Secondly, in order to determine the effect of salivary thiocyanate on the formation of NDMA, experiments were performed in which codfish and spinach were added to the gastric compartment, with or without thiocyanate. Thiocyanate (2 mM) was continuously added to the gastric compartment (6 ml/h) simulating the swallowing of oral fluid (saliva) containing thiocyanate. In total six experiments were performed with two batches of codfish.

Thiocyanate is normally present in saliva, therefore further experiments were performed in the presence of 2 mM thiocyanate. The inhibition of NDMA formation was investigated by introducing codfish and spinach into the gastric model together with a putative nitrosation inhibitor. Codfish (70 g) and spinach (50 g) mixed with artificial saliva and gastric juice were added to the model with or without 80 ml of orange juice (containing 35 mg ascorbic acid). The same experiment was performed in which orange juice was replaced by 80 ml black tea (0.5 g lyophilized tea extract dissolved in boiling water). Furthermore, experiments were performed in which orange juice or black tea, was loaded into the model 1 h after the start, simulating the effect of the intake of inhibitors after the meal.

Nitrite was gradually added, simulating the intake of two times the ADI of nitrate.

2.4.6. Flow of nitrite-containing oral fluid, quantification based on toxicokinetic model

A toxicokinetic model of nitrate and nitrite has been developed and will be described elsewhere (Zeilmaker et al., in preparation). This model is based and verified on various studies in humans, including the time-course of the concentrations of nitrate and nitrite in plasma and oral fluid after the intake of nitrate-containing vegetables and drinking water.

The concentration curves of nitrite in oral fluid were calculated by the model simulating the intake of 0.1, 1, 2, 5 and 10 times the ADI of nitrate. The oral fluid concentration of nitrite increased approximately linearly, almost immediately after the intake of nitrate. A maximum was reached at around 1 h; thereafter the concentration of nitrite declines linearly (example 10 times ADI of nitrate, Fig. 3).

The swallowing of nitrite-containing oral fluid according to above calculated curves was mimicked by continuously infusing a solution of nitrite (32 mM) into the gastric compartment of the gastrointestinal model during the 3 h experiment, at the following flow-rates: 1 ml/h for the first 15 min, 3.5 ml/h between 15 and 30 min, 6.9 ml/h between 30 and 45 min, 10.5 ml/h between 45 and 60 min, 11.7 ml/h between 60 and 120 min, and 10.7 ml/h between 120 and 180 min. Similar procedures were performed to simulate the intake of 0.1, 1, 2 and 5

times the ADI of nitrate, resulting in total nitrite flows during 3 h of 0.028, 0.072, 0.105, and 0.45 mM (Fig. 4). In this way we simulated the swallowing of nitrite-containing oral fluid in humans after the intake of 0.1–10 times the ADI of nitrate.

2.5. Statistics

To describe the dose–response curve between the amount of nitrite added to the model and the amount of NDMA formed in the gastric compartment, the log likelihood ratio test was used to determine the parameters for the best fit of the data ($P<0.05$; Advanced Continuous Simulation Language, Edition 10.1, Mitchell and Gauthier Associates, Concord, MA 01742, USA). The Student t-test was used to evaluate the statistical significance of the observed differences between experiments with or without thiocyanate (P-value of 0.05).

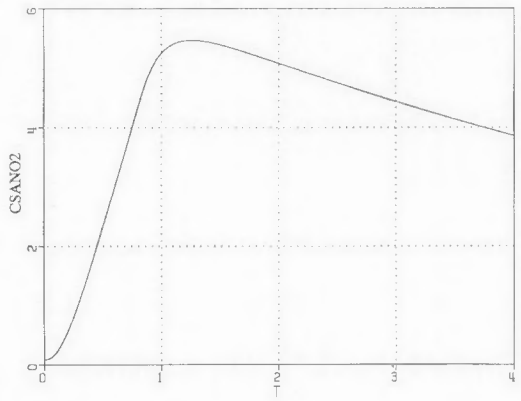

Fig. 3. Model simulation of the time-course of the concentration of nitrite in human oral fluid after exposure to 10 times the Acceptable Daily Intake of nitrate, i.e. 36.5 mg nitrate/kg body weight. X-axis: T = time in hours; Y-axis: Csano2 = concentration of nitrite in oral fluid (mM).

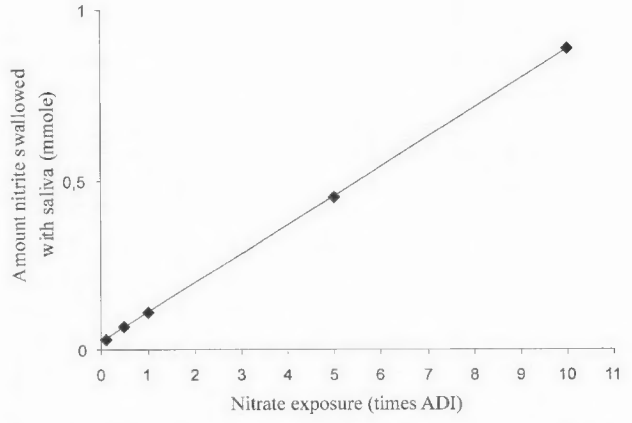

Fig. 4. Model simulation of the total amount of nitrite swallowed with oral fluid after the exposure to 0.1 to 10 times the Acceptable Daily Intake of nitrate (0.36–36.5 mg nitrate/kg body weight).

3. Results

3.1. NDMA formation measured with DMA and a fixed dose of nitrite

In general, more NDMA was formed during the 3-h gastric passage when the gastric pH decreased rapidly, compared to the NDMA formation during the slow gastric pH decrease (Fig. 5). Under the conditions of a rapid gastric pH decrease the majority of NDMA was produced between 60 and 120 min after the start of the experiment. During this time interval the pH in the gastric compartment decreased from 2.5 to 1.7. When the pH decreased more slowly, most of the NDMA was formed between 120 and 180 min, during which time the pH decreased from 3 to 1.7. Relative high cumulative amounts of NDMA were produced (mean 128 μg; range 113–143 μg) after the intake of 5 mM nitrite and 5 mM DMA, under the conditions of a rapid gastric pH decrease. Lower cumulative amounts of NDMA were formed (mean 39 μg; range 35–42 μg) under the conditions of a slow gastric pH decrease. Under the conditions of a rapid gastric pH decrease, the formation of NDMA was only weakly affected by the concentration of its precursor DMA. A lower concentration of DMA (2.5 mM) did not significantly change the cumulative amount of the NDMA formed compared to 5 mM DMA (data not shown).

In the control experiments with 5 mM DMA without nitrite, cumulative amounts of 7.8 and 14.3 μg NDMA were measured, under conditions of slow and rapid gastric pH decrease, respectively. The explanation is probably contamination of the DMA solution with a small amount of NDMA. In the control experiments

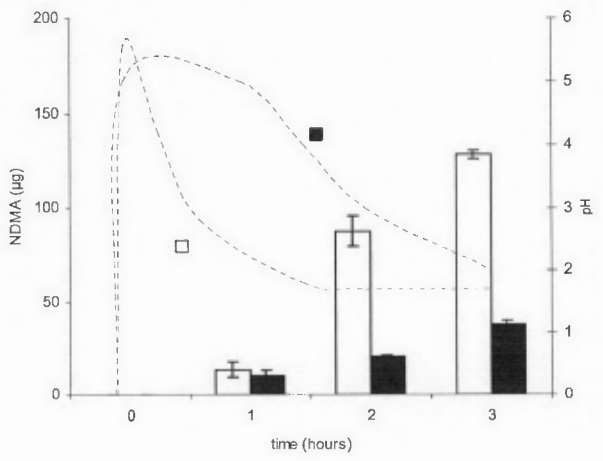

Fig. 5. The cumulative amount (mean ± range; $n=2$) of NDMA formed in the gastric compartment after the intake of 5 mM DMA and 5 mM nitrite, performed under conditions of a rapid gastric pH decrease (white columns) and a slow gastric pH decrease (black columns). The dotted curves show the pre-set pH in the gastric compartment.

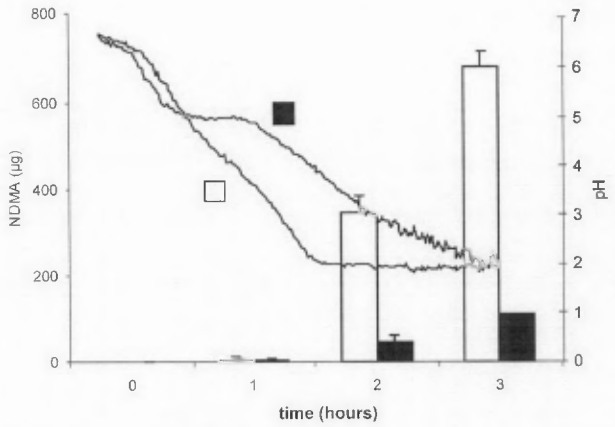

Fig. 6. The cumulative amount (mean±range; $n=2$) of NDMA formed in the gastric compartment after the intake of 100 g of codfish and 5 mM nitrite, performed under conditions of a rapid gastric pH decrease (white columns) and a slow gastric pH decrease (black columns). The curves show the pH measured in the gastric compartment.

without any of the precursors 1.2–1.4 µg NDMA was detected.

In conclusion, this first set of experiments showed that NDMA was produced in the gastric compartment when nitrite and DMA are introduced into the model as pure compounds.

3.2. NDMA formation measured with codfish and a fixed dose of nitrite

The addition of codfish as a source of amines appeared to influence the pH in the gastric compartment (Fig. 6). Consequently, the pH did not exactly follow the pre-set points of the curve for rapid gastric pH decrease (compare Figs. 6 and 2).

Higher amounts of NDMA were formed when codfish was used instead of 5 mM DMA together with 5 mM nitrite. The cumulative amount of NDMA produced was 677 µg (range 669–684 µg) and 109 µg (range 90–128 µg), under conditions of a rapid and slow gastric pH decrease, respectively. Apparently DMA from codfish became available later in time, at the time the pH reached a level that favours the nitrosation reaction, and thus resulted in higher amounts of NDMA (compared to the DMA solution).

In the control experiments in which codfish was added to the model without nitrite 1.6±1 µg ($n=3$; range 0.9–2.8 µg) NDMA was produced (Table 1). Apart from NDMA, a very small amount of nitrosodiethylamine (NDEA) was found in the GC-TEA chromatogram of some of the samples derived from the in vitro model.

3.3. NDMA formation measured with codfish and gradual addition of nitrite

The gastric compartment was loaded with 5 mM DMA while the addition of nitrite simulated the swallowing of nitrite-containing oral fluid after the intake of 10 times the ADI of nitrate. The mean cumulative amount of NDMA formed under the conditions of a rapid and slow gastric pH decrease was 106 µg (range 65–146 µg) and 39 µg (range 19–58 µg), respectively (Fig. 7). The cumulative amount of NDMA was equal to the amount formed after the intake of a mixture of 5 mM DMA and 5 mM nitrite at once (for both the rapid and slow gastric pH decrease). However, differences were seen in the time course of NDMA formation. Most of the NDMA was produced between 120 and 180 min. This is in contrast to the results of the experiments in which nitrite was added as a fixed dose, where NDMA formation occurred largely between 60 and 120 min (compare Figs. 5 and 7).

In the experiments with nitrite at the level of 10 times the ADI of nitrate in combination with 100 g of codfish, the mean cumulative amount of NDMA was 423 µg (range 292–554 µg) and 43 µg (range 41–44 µg) under the conditions of a rapid and slow gastric pH decrease, respectively (Fig. 8). These amounts of NDMA were less than produced in the experiments in which codfish and 5 mM nitrite were given at once (Table 1).

Table 1
The cumulative amount of N-nitrosodimethylamine (NDMA) formed in the gastric compartment of the gastrointestinal model after the intake of nitrosation precursors ($n=2$; except were no range is given)

Intake		Rapid pH decrease NDMA (µg)		Slow pH decrease NDMA (µg)	
Nitrite (mM)	DMA (mM)	Mean	Range	Mean	Range
–	–	1.4	–	1.2	–
–	5	14.3	–	7.8	–
–	100 g codfish	1.1	0.9–1.2	2.8	–
5	5	128	113–143	39	35–42
5	100 g codfish	677	669–684	109	90–128
10×ADI nitrate	5	106	65–146	39	19–58
10×ADI nitrate	100 g codfish	423	292–554	43	41–44
5×ADI nitrate	100 g codfish	–	–	21	14–28
1×ADI nitrate	100 g codfish	16.5	14.7–18.2	5.1	4.7–5.5
0.1×ADI nitrate	100 g codfish	2.3	1.6–2.9	1.8	–

In addition, experiments were performed with 100 g of codfish and the addition of nitrite, simulating the intake of 0.1, 1 and 5 times the ADI of nitrate. All experiments were performed under the conditions of a rapid and slow gastric pH decrease (except for five times the ADI of nitrate, which was only performed under the conditions of a slow gastric pH decrease). At 0.1 and 1 time the ADI of nitrate, 2.3 and 16.5 μg NDMA were formed, under the conditions of a rapidly decreasing gastric pH (Table 1). At 0.1, 1 and 5 times the ADI of nitrate, 1.8, 5.1 and 21.1 μg NDMA were formed under the conditions of a slowly decreasing gastric pH (Table 1).

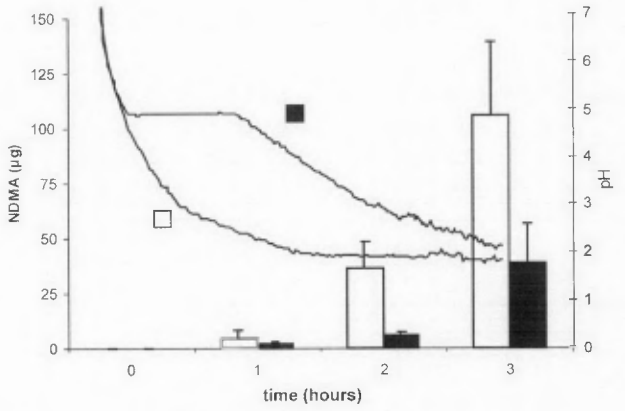

Fig. 7. The cumulative amount (mean±range; $n=2$) of NDMA formed in the gastric compartment after the intake of 5 mM DMA and the gradual addition of nitrite (continuously from 0 to 3 h), simulating the swallowing of nitrite-containing oral fluid after the intake of 10 times the Acceptable Daily Intake of nitrate. The experiments were performed under conditions of a rapid gastric pH decrease (white columns) and a slow gastric pH decrease (black columns). The curves show the pH measured in the gastric compartment.

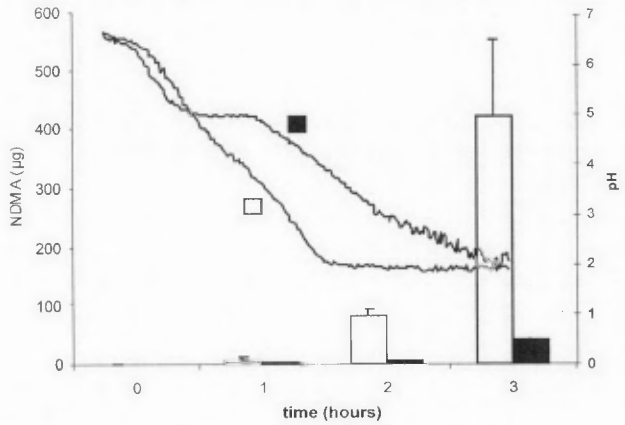

Fig. 8. The cumulative amount (mean±range; $n=2$) of NDMA formed in the gastric compartment after the intake of 100 g of codfish and the gradual addition of nitrite (continuously from 0 to 3 h), simulating the swallowing of nitrite containing oral fluid after the intake of 10 times the Acceptable Daily Intake of nitrate. The experiments were performed under the conditions of a rapid gastric pH decrease (white columns) and a slow gastric pH decrease (black columns). The curves show the pH measured in the gastric compartment.

3.4. NDMA formation measured with various fish species and batches

The cumulative formation of NDMA (mean±S.D.) in the gastric compartment during 3 h after the intake of various species and batches of fish (100 g portions) and the addition of nitrite simulating the intake of five times the ADI of nitrate, ranged from 0 to 235 μg (Table 2). The mean amount of NDMA produced was the highest for codfish (194±32 μg), followed by herring (76±29 μg), pollack (32±21 μg), and plaice (28±8 μg). The cumulative amount of NDMA formed after introduction of samples derived from mackerel, salmon and pike perch was around the background level, i.e. the level of NDMA formed or present in the model without the introduction of one or both of the precursors (Table 2).

The results obtained with codfish and the addition of nitrite simulating the intake of five times the ADI of nitrate, together with the results obtained with codfish and nitrite simulating the intake of 0.1, 1 and 10 times the ADI of nitrate, were used to analyse the dose–response relation between the amount of nitrite added to the model and the amount of NDMA formed in the gastric compartment. Under the condition of a rapid gastric pH decrease, a non-linear model is to be preferred over a linear model in describing the dose–response relation (log likelihood ratio test, $P<0.05$; Fig. 9).

3.5. Modulation of NDMA formation, influence of thiocyanate and antioxidants

The mean cumulative formation of NDMA after the intake of 70 g of codfish and the addition of nitrite, simulating of the intake of 2 times the ADI of nitrate, was 63 μg (range 53–74 μg). Simulating a more realistic meal intake with spinach (50 g) and codfish (70 g) in

Table 2
The cumulative amount of N-nitrosodimethylamine (NDMA) formed in the gastric compartment of the gastrointestinal model after the intake of various fish species (100 g) combined with the addition of nitrite simulating the intake of five times the ADI of nitrate

Fish species	No. of batches	NDMA (μg) mean±S.D.	NDMA (μg) range
Codfish	5	194±32	157–235
Herring	5	76±29	45–120
Pollack	4	32±21	14–62
Plaice	4	28±8	23–39
Mackerel	4	3.2±1.7	1.7–5.7
Salmon	4	1.2±2.5	0–4.9
Pike perch	1[a]	1.5±0.8	0.9–2.1
Herring	1[b]	0.6	
Citrate buffer	1	0.8	

[a] With the addition of nitrite simulating the intake of 10 times the ADI of nitrate.
[b] Without the addition of nitrite.

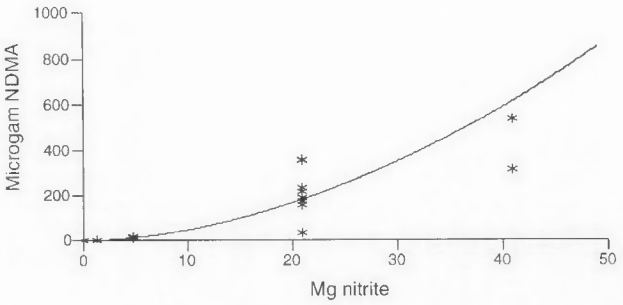

Fig. 9. Dose–response curve ($y=0.72\ x^{1.82}+1.47$) between the amount of nitrite added to the model and the amount of NDMA formed in the gastric compartment, simulating a rapid gastric pH decrease.

combination with nitrite simulating two times the ADI of nitrate, substantially lowered the NDMA formation (7.2±3.4 μg; range 3.7–13 μg, $n=6$). Previously, in a pilot experiment, we observed that spinach (75 g) added to codfish (100 g) and nitrite simulating the intake of 10 times the ADI of nitrate, substantially lowered the cumulative amount of NDMA from 423 μg (range 291–555 μg) to 159 μg (range 125–189 μg).

The role of thiocyanate was determined in an experiment performed under the same conditions as described above; 70 g of codfish, 50 g spinach and nitrite simulating the intake of two times the ADI of nitrate. When thiocyanate (2 mM) was continuously added to the gastric compartment, simulating the swallowing of oral fluid, the total formation of NDMA was increased from 7.2±3.4 μg (without thiocyanate) to 10.6±5.2 μg (range 5.5–19.5 μg, $n=6$). Although, the mean NDMA formation was increased with 47%, the difference was not statistically significant (Table 3).

In contrast to the increase of NDMA observed by the addition of thiocyanate, introducing antioxidants into the meal with codfish, spinach and nitrite (simulating two times the ADI of nitrate) inhibited the NDMA formation. Eighty milliliters of orange juice (containing 35 mg ascorbic acid) added to the meal intake before introduction into the model, resulted in the cumulative formation of 4.5 μg NDMA (range 3.9–5.1 μg). Orange juice introduced into the gastric compartment 1 h after the intake of the meal with spinach and codfish, resulted in 4.6 μg NMDA (range 4.3–4.8 μg).

Similar results were obtained for black tea (0.5 g dissolved in boiling water): 3.2 μg NDMA (range 3.0–3.4 μg) and 3.0 μg NDMA (range 2.9–3.0 μg) was formed, when black tea was given together with the meal or 1 h after the intake of the meal, respectively. Thus, the addition of antioxidants simultaneously or one hour after the meal intake (codfish and spinach) did result in a similar inhibition of NDMA formation; 4.5 μg and 3.0 μg for orange juice and tea, respectively, compared to 10.6 μg NDMA under the same conditions without these inhibitors (Table 3).

4. Discussion

The present study shows that substantial amounts of NDMA are formed in the gastric compartment of the dynamic in vitro gastrointestinal model in the presence of its precursors, a variety of fish species in combination with nitrite, under defined conditions simulating the human physiological situation in the stomach.

For the risk evaluation of NDMA exposure after consumption of fish and nitrate-containing vegetables, it would be useful to predict the level of NDMA formation based on the concentrations of amines present in fish. However, this seems to be hardly possible. No strict correlation was found between the individual concentrations of amines, such as dimethylamine (DMA), trimethylamine and trimethylamine oxide, measured in batches of codfish and herring used in this study and the formation of NDMA (M. Kotterman, personal communication, RIVO). Nevertheless, it is clear that to some extent DMA or enzymes responsible

Table 3
The cumulative amount of N-nitrosodimethylamine (NDMA) formed in the gastric compartment of the gastrointestinal model after the intake of nitrosation precursors and modulators ($n=2$)

Intake					NDMA (μg)	
Nitrite	Codfish (g)	Spinach (g)	Thiocyanate (mM)	Antioxidant	Mean	Range
10×ADI nitrate	100	–	–	–	423	291–555
10×ADI nitrate	100	75	–	–	159	125–189
2×ADI nitrate	70	–	–	–	63	53–74
2×ADI nitrate	70	50	–	–	7.2±3.4	3.7–13[a]
2×ADI nitrate	70	50	2	–	10.6±5.2	5.5–19.5[a]
2×ADI nitrate	70	50	2	Orange juice (80 ml)	4.5	3.9–5.1
2×ADI nitrate	70	50	2	Orange juice (80 ml)[b]	4.6	4.3–4.8
2×ADI nitrate	70	50	2	Black tea (0.5 g)	3.2	3.0–3.4
2×ADI nitrate	70	50	2	Black tea (0.5 g) [b]	3.0	2.9–3.0

[a] $n=6$.
[b] Added 1 h after the intake of codfish and spinach.

for the conversion of amines into DMA are necessary precursors for NDMA formation. Pike perch, as an example of a non-gadoid fish species, and grilled steak tartar, as an example of meat (data not shown), did not result in NDMA formation above the background level.

The formation of NDMA from precursors with in vitro models was studied earlier under specific static conditions, such as a fixed gastric pH and unrealistically high concentrations of nitrite (Walker, 1990). A few studies were performed with food products, among which different types of fish, mimicking the human situation by addition of artificial saliva and gastric juice (Groenen et al., 1982; Sen et al., 1985). These static in vitro experiments do not reflect the kinetic conditions in the human stomach. In our study the pH profile in the dynamic in vitro gastrointestinal model was set to simulate the human physiological situation in the stomach: an initial incubation at pH 5 (immediately after ingestion) and then a gradual decrease to pH 1.7.

Direct comparison between static models and our dynamic model is difficult. In our experiment under conditions of a rapid gastric pH decrease, the amount of NDMA formed was 16.5 μg (range 14.7–18.2 μg) simulating the intake of 100 g of codfish and one times the ADI of nitrate (total mass flow of 0.105 mM nitrite). This is in the same range as observed by Groenen et al. (1980, 1982). They found 6–18 μg NDMA after 2 h of incubation at pH 2 with 250 g of codfish and 0.15 mM nitrite. In our experiments, however, the concentrations of the precursors in the gastric compartment at the time the gastric pH was two, were much lower than in the studies of Groenen et al. (1982). Thus, quantitative comparison showed that in our in vitro model approximately the same amounts of NDMA were formed using lower concentrations of the precursors.

Apart from the lack of pH changes in the static systems, another important shortcoming of the static models is that nitrite is added directly to the incubation mixture, whereas the actual uptake of nitrite via food is usually negligible. The nitrite concentration starts to increase by active secretion of nitrate in the saliva, approximately 1 h after the intake of nitrate. To predict the nitrosation more precisely, the nitrite concentration in our model was programmed to increase slowly after the intake of fish, reaching a peak value after 1 h. Higher concentrations of nitrite were reached in the gastric compartment when the rate of gastric emptying decreases, thereby increasing the efficiency of the reaction of nitrite with DMA. In conclusion, the dynamic in vitro gastrointestinal model simulating human digestion mimics the in vivo nitrosation probably better than static models.

In contrast to in vivo studies, the dynamic in vitro gastrointestinal model presented here offers the opportunity to investigate the formation of carcinogenic nitrosamines, such as NDMA, over time in the gastric compartment under realistic conditions. Research on the process of nitrosation in the human stomach with the use of food matrices is difficult and not ethical. Therefore human studies are usually limited to analysis of excretion products in the urine, e.g. performed with the N-nitrosoproline (NPRO) assay. However, in human studies no correlation was found between N-nitrosoproline excretion and dietary nitrate intake (Tannenbaum, 1987).

On the basis of NDMA excretion in the urine, Vermeer et al. (1998) estimated the amount of NDMA produced in the stomach at 174 μg per day. In our study 16.5 μg NDMA were formed, simulating approximately the same conditions as in the human study of Vermeer et al. However, urinary NDMA excretion in the human study reflects the repeated daily exposure to a fish meal and nitrate at the level of one times the ADI, while in our experiment the amount of NDMA was formed after a single exposure (viz. the intake of 100 g codfish and nitrite at the level corresponding to one times the ADI of nitrate). Besides, N-nitrosamines are rapidly and almost completely metabolised, estimations of the NDMA formation rates in the human stomach on the basis of urinary excretion may not be accurate. Another explanation for the difference in NDMA formation could be that part of the NDMA was formed at other sites in the body by non-acid-catalysed mechanisms, as described by Leaf et al. (1989, 1990). This extra-gastric NDMA formation is obviously not detected in our gastric model.

It should be mentioned that the dynamic in vitro model does not take into account the gastric absorption of nitrite. Licht et al. (1986) and Licht and Deen (1988) argued that nitrite absorption in the human stomach, rather than gastric emptying and dilution by food consumption is the main determining factor for NDMA formation. This argument was based on observations in the stomach of fasting dogs, i.e. circumstances that may facilitate nitrite absorption. In humans, NDMA formation occurs in a stomach that contains food, i.e. under circumstances that should inhibit absorption. This means that the permeability rate of the human stomach for nitrite may be quite different from the rate that is prevalent in the empty stomach of dogs. Mirvish et al., indeed showed that nitrite loss in the stomach of rats is much slower when rats were given food mixed with nitrite, compared to a liquid nitrite solution (Mirvish et al., 1975). Unfortunately there are no human data available that can be used to evaluate the effect of the food matrix on the absorption of nitrite in the stomach. On the other hand, nitrite excretion by the stomach mucosa can neither be excluded.

Furthermore, the gastric nitrite concentration might have been lowered in humans by the presence of antioxidants, such as ascorbic acid or tea polyphenols in the gastric juice. The results obtained in our gastrointestinal

model showed that food compounds, such as orange juice and black tea effectively inhibited, but not totally prevented the nitrosation reaction under physiological conditions. Nitrosation inhibitors usually react with nitrosating agents. Therefore they act as competitors for amines, which are substrates for the nitrosating agents. Vermeer et al. (1999) presented the effects of ascorbic acid on urinary excretion of NDMA in human volunteers. They showed that the urinary NDMA excretion during the intervention with fish, nitrate and 250 mg ascorbic acid was decreased to the background levels. A four times higher dose of ascorbic acid did not show an additional effect on the inhibition of urinary NDMA excretion. Other studies in humans also reported no effect of ascorbic acid on the basal NDMA and NPRO excretion (Leaf et al., 1987; Garland et al., 1986; Wagner et al., 1985). Likewise, in our model, at higher levels of NDMA formation (by the introduction of 100 g of codfish and nitrite mimicking the intake of 10 times the ADI of nitrate), the reduction of nitrosation by 250 mg ascorbic acid was approximately the same as observed in the experiments with orange juice, namely 60% (data not shown). In addition, under the same conditions the NDMA formation could not totally be reduced at higher dose levels of ascorbic acid (up to 1000 mg).

The results presented in this study also showed that spinach reduced the formation of NDMA considerably. The preventive mechanism could be related to the reduction of nitrite into NO, which is not a directly reactive compound, by ferridoxin nitrite reductase or ascorbic acid present in spinach (Mikami and Ida, 1989; Gill et al., 1999). Our results suggest that relatively small amounts of nitrosation inhibitors present in fruit and vegetables are sufficient to considerably reduce, but not completely prevent, the NDMA formation. A human intervention study indeed showed that the amount of ascorbic acid and other inhibitors present in the daily intake of vegetables and fruit was not sufficient to prevent the endogenous formation of NOC (Vermeer et al., 1998).

The formation of NDMA was also effectively inhibited by black tea extracts (0.5 g) added to the food intake of the model. Similar to the results obtained with ascorbic acid, NDMA formation could not totally be reduced, even not at higher dose levels (up to 4 g of green or black tea; data not shown). In the human study by Vermeer et al., 4 g of green tea resulted in even increased NDMA excretion (Vermeer et al., 1999). Those results suggest that increasing the ratio between nitrite and nitrite-reducers does not always result in a progressively stronger inhibition of NDMA formation. The dose–effect relationships and the effectiveness of NOC inhibitors need further investigation.

Several studies have indicated that dietary nitrate exposure increases the nitrite concentration in the saliva. The reaction between salivary nitrite and ascorbic acid into NO is extremely rapid, but NO is also rapidly recycled to nitrite in the presence of oxygen (Mowat et al., 1999). Swallowing is the major source of delivering oxygen into the stomach. Therefore, oxygen will enhance ascorbic acid consumption and increase nitrosation at the side where salivary nitrite enters acidic gastric juice (Moriya et al., 2002). The pH in gastro oesophageal junction and the cardia of the stomach favours nitrosation, because it escapes the buffering effect of food (Fletcher et al., 2001). The anatomical site where acid nitrosation is maximal corresponds with the increasing incidence of mutagenesis and carcinogenesis at the gastro oesophageal junction and cardia (Hansson et al., 1993; Hansen et al., 1997; Mayne et al., 2001).

It is evident that the endogenous formation of NDMA depends on many factors, including the pH in the gastric compartment, the concentrations of secondary amines, nitrate, nitrite, thiocyanate and nitrosation inhibitors. Information on the intragastric formation of NDMA from complex mixtures of precursors and inhibitors in humans is not available. It is important to investigate NDMA formation under various physiological conditions present in the human gastrointestinal tract. The dynamic in vitro gastrointestinal model offers the possibility to follow the NDMA formation in time at the location where it is formed. In this model the cumulative amount of N-alkylnitrosamines formed can be quantified, in contrast to the human body in which N-alkylnitrosamines are quickly distributed and metabolised. The present study clearly demonstrates that rather high amounts of NDMA are formed under physiological gastric conditions, if both appropriate precursors are present. The results are supported in experiments using more complex food matrices, such as nitrate-containing vegetables, various species of fish and food-related nitrosation inhibitors. The results show that the model seems to be a useful tool to investigate mechanistically the NDMA formation, under human conditions without ethical and practical limitations.

IARC has classified various N-alkylnitrosamines as probably or possibly carcinogenic to humans. Results obtained with this model are helpful in evaluating possible human cancer risks for long-term exposure (e.g. to frequent low levels) or to incidental exposure (e.g. to high levels) to nitrate containing vegetables in combination with gadoid (codfish-like) fish. Exposure to endogenous NDMA formation turned out to be much higher than exposure to exogenous NDMA. This indicates that risk assessment for nitrate intake should be focused not only on the toxicity of nitrate itself, but also on its role as substrate for the formation of one of the NDMA precursors. In the absence of extensive quantitative risk estimation so far, one may be well-advised to reduce exposure to exogenous N-nitrosamines and to

be careful with combined intake of the precursors of N-nitrosamines.

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1335 ABDOMINAL INJURY-HOCKEY

T.J. Jagielo, Lutheran General Hospital, Park Ridge, IL
(Sponsor: W.W.Briner, Jr., FACSM)

HISTORY - A 28-year-old male was checked over the boards and sustained a contact injury to his right lower abdominal region. The patient complained of right lower abdominal pain throughout the remainder of the game but was able to continue playing. Approximately 45 minutes after the game, the patient developed light-headedness, diaphoresis and worsening right lower abdominal pain. The patient passed several grossly bloody stools during the course of the night but did not seek medical attention. The following morning he continued to have right lower quadrant pain and several bloody stools, consequently seeking attention in the Emergency Department. The patient denied history of hematemesis, peptic ulcer disease, inflammatory bowel disease, prior melena or grossly bloody stools.

PHYSICAL EXAMINATION - In the emergency department the patient was in no apparent distress. The pulse rate was 79 with no significant change in pulse or blood pressure with standing. There was no costovertebral tenderness. The abdomen was nondistended, normoactive bowel sounds were present and there was tenderness with rebound in the right lower quadrant. There was no rigidity or guarding. Rectal was nontender with grossly bloody stool. Extremity exam revealed strong pulses with warm and dry skin.

ABDOMINAL INJURY-HOCKEY
T.J. Jagielo, Lutheran General Hospital, Park Ridge, IL
(Sponsor: W.W. Briner, Jr., FACSM)

DIFFERENTIAL DIAGNOSIS:
1. Right colonic contusion with intraluminal hemorrhage
2. Perforated viscus
3. Small bowel contusion with intraluminal hemorrhage

TESTS AND RESULTS:
NG-tube aspirate was bilious and non-bloody. Amylase, lipase, LFTs, PT, PTT, electrolytes and UA were normal. Hgb 16.1 and platelets 257. WBC was 9.0 with normal differential. Abdominal obstructive series did not reveal free air. C.T. of the abdomen and pelvis revealed a cecal to hepatic flexure colonic wall thickening consistent with an intramural hematoma.

FINAL/WORKING DIAGNOSIS:
Cecal to right hepatic flexure colonic intramural hematoma

TREATMENT:
1. IVF, NPO, Type and cross PRBC for possible transfusion, serial vital sign and hemogram monitoring.
2. Surgery consultation for emergent surgery for viscus perforation or hemodynamic instability secondary to continued hemorrhage.

The patient was hydrated with IVF and kept NPO. Transfusion was unnecessary because of hemodynamic stability throughout hospitalization and stable serial hemograms. After 24 hour observation the diet was advanced and the patient subsequently discharged home with recommended follow-up in one week.

1336 PAINLESS HEMATURIA - FOOTBALL

P.M. Johnson, M.D.
St. Joseph's Medical Center, South Bend, IN
(Sponsor: J.M. Moriarty, M.D. FACSM, Univ. Notre Dame)

HISTORY - A 19-year-old intramural football player presentad to the clinic with a one day history of painless hematuria. While playing quarterback in a contact football game, he sustained numerous hits to the thorax and back region. He had no back or abdominal pain after the football game. After an evening of drinking, he noted gross hematuria on three occasions during the night. He presented to the clinic in the afternoon after more episodes of gross hematuria. He described the urine as dark red and denied any back pain or dysuria or previous episodes of hematuria.

PHYSICAL EXAMINATION - Examination 24 hours after the incident revealed no CVA tenderness or flank pain. Patient had tenderness on his right side at ribs seven through nine along the mid-axillary line. There was no abdominal pain, tenderness, guarding or palpable masses. He had no suprapubic tenderness or other pelvic findings. The patient's musculoskeletal and neurological examination was unremarkable with normal range of motion of the spine and lower extremities. Patient's blood pressure was similar to other clinic visits.

PAINLESS HEMATURIA - FOOTBALL
P.M. Johnson, M.D.
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(Sponsor: J.M. Moriarty, M.D. FACSM, Univ. Notre Dame)

DIFFERENTIAL DIAGNOSIS:
1. Ureteral laceration
2. Myoglobinuria
3. Bladder hemorrhage
4. Acute renal hemorrhage
5. Polycystic kidney disease
6. Renal obstruction or nephrolithiasis
7. Renal vascular anomaly

TEST AND RESULTS:
1. Complete blood count:
   - Hemoglobin 15.3, platelets 223,000.
2. Urinalysis:
   - gross hematuria, innumerable red blood cells
3. CT Scan abdomen and pelvis:
   - Congenital UPJ obstruction, right kidney.
   - Acute hemorrhage with hematoma within the right renal pelvis.

FINAL/WORKING DIAGNOSIS:
Acute hemorrhage/hematoma right renal pelvis with congenital UPJ obstruction.

TREATMENT:
1. Hospital observation.
2. Resolution of gross hematuria four days after contact.
3. Urologic recommendations.
4. Return to contact/non-contact sports?

1337 RENAL INSUFFICIENCY AFTER CREATINE SUPPLEMENTATION IN A COLLEGE FOOTBALL ATHLETE

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HISTORY - A 19-year old Division I football player presented with complaints of fatigue and dyspnea during football practice. He first noticed symptoms one month prior to his examination with progressively worsening complaints and an associated ten pound weight loss. Past history includes asthma treated with inhaled beta-agonists, and no other medial problems (e.g., diabetes, musculoskeletal complaints, rash, or renal disease). Medications: albuterol and nedocromil inhalers, zafirlukast tablets, with no history of anabolic steroid use or non-steroidal antiinflammatory drug use. The patient reported taking 10 grams of creatine monohydrate supplement each day for the past three months.

PHYSICAL EXAMINATION - Blood pressure (BP):116/68 mmHg, Heart rate (HR): 72 beats/min. Temperature: 37.1°C. Weight 116 kg. No orthostatic HR or BP change, no pallor. Cardiovascular (CV): Regular rate and rhythm without murmurs. Lungs: Faint end expiratory wheezing right base. The rest of the physical examination was normal.

RENAL INSUFFICIENCY AFTER CREATINE SUPPLEMENTATION IN A COLLEGE FOOTBALL ATHLETE
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LABORATORY TESTS: Serum Creatinine =1.7; HCT = 39.5 and MCV = 82.9 FL; CrCl =138 ml/min.; BUN = 18; K^+, Na^+ Normal. CK = 996 U/L; Urinalysis = normal. SPIROMETRY: FEV1 = 4.44 L (87%), FVC = 5.28 L (89%).

TREATMENT: The patient was placed on aerosolized steroids, continued on prn beta agonists and creatine supplementation was discontinued. No other medications were used and he continued football practice.

COURSE: Serum Cr slowly decreased to 1.3 over a one month period, after discontinuing creatine supplementation. Fatigue and dyspnea resolved.

ASSESSMENT: To our knowledge, this is the first report of renal insufficiency induced by regular creatine supplementation. The use of this supplement, especially in athletes who regularly use potential nephrotoxins such as NSAIDS, should have their renal function assessed. Further examination of the potential adverse effects of creatine should be examined.

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Protective effect of dietary nitrate on experimental gastritis in rats

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Nitrates have long been considered as harmful dietary components and judged responsible for deleterious effects on human health, leading to stringent regulations concerning their levels in food and water. However, recent studies demonstrate that dietary nitrate may have a major role in human health as a non-immune mechanism for host defence, through its metabolism to NO in the stomach. NO is a versatile molecule and although evidence exists showing that administration of low doses of exogenous NO protects against gastrointestinal inflammation, higher NO doses have been shown to exacerbate injury. So, the effect of an ingestion of nitrates in doses corresponding to a normal diet in human consumers on an experimental gastritis induced by iodoacetamide in rats was investigated. During gastritis one of the following compounds was given orally: water; KNO_3; the NO donor sodium nitroprusside; the NO scavenger haemoglobin given with either water or KNO_3. N(G)-nitro-L-arginine methyl ester (L-NAME), a non-specific NO synthase inhibitor, was administered with either water, iodoacetamide alone, or combined with KNO_3. After killing, the stomach was resected and microscopic damage scores, myeloperoxidase and NO synthase activities were determined. Iodoacetamide-induced gastritis was significantly reduced by KNO_3 administration, an effect which was reproduced by sodium nitroprusside and reversed by haemoglobin. L-NAME induced gastric mucosal damage in itself, and KNO_3 did not prevent the gastritis induced by iodoacetamide associated with L-NAME. In conclusion, dietary nitrate exerts a protective effect against an experimental gastritis in rats by releasing NO in the stomach but such an effect requires the production of endogenous NO.

Nitric oxide: Inflammation: Dietary nitrate: Stomach

The human diet is a source of direct (natural components) and indirect (additives, pesticides, etc) factors which are able to modulate gastrointestinal functions. Among these potentially modulating dietary factors, nitrates occupy a controversial position. The main sources of nitrates for human consumers are green and leafy vegetables, drinking water and also some meat and fish products where nitrates are widely used as preservatives in the form of Na and K salts. The total dietary intake of nitrates, excluding nitrates in the water, has been estimated as 50–80 mg/d per person (Meah *et al.* 1994; Dich *et al.* 1996). When ingested, dietary nitrate is absorbed from the stomach and proximal intestine into the plasma and then concentrated in saliva (Tannenbaum *et al.* 1976), where it is rapidly reduced to nitrites by anaerobic bacteria (Spiegelhalder *et al.* 1976; Walters & Smith, 1981). The two potentially deleterious effects of high gastric concentrations of nitrates are methaemoglobinaemia (Craun *et al.* 1981) and formation of carcinogenic N-nitroso compounds (Bruning-Fann & Kaneene, 1993). However, beyond these potential effects, nitrites are transformed into NO under the acidic conditions of the stomach (Benjamin *et al.* 1994; Duncan *et al.* 1995), an event that has been shown to participate in the protection of the organism against pathogen-induced

Abbreviations: cNOS, constitutive NO synthase; EGTA, ethylene-glycol-bis(a-aminoethyl)-N,N,N′,N′-tetra-acetic acid; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; iNOS, inducible NO synthase; L-NAME, N(G)-nitro-L-arginine methyl ester; MPO, myeloperoxidase; NOS, nitric oxide synthase; SNP, sodium nitroprusside.

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gastrointestinal and oral diseases (Green, 1995; Dykhuizen *et al.* 1996; Duncan *et al.* 1997). In human subjects, ingestion of small amounts of nitrates (i.e. 2 mmol which corresponds to the amount normally found in a lettuce; Meah *et al.* 1994) has thus been found to generate high concentrations of NO in the stomach (McKnight *et al.* 1997).

Gastric NO may not be delivered only by extrinsic sources, but may also be generated by mucosal cells, which are able to produce large amounts of NO (Konturek & Konturek, 1995). Indeed, endogenous NO is produced from the amino acid L-arginine by either constitutive NO synthase (cNOS) or inducible NO synthase (iNOS) (Moncada *et al.* 1991) and both NOS have been detected in homogenates of rat gastric mucosa (Tepperman & Soper, 1994). In physiological conditions, cNOS is the only one expressed in gastric mucosa where it plays an important role in the maintenance of mucosal integrity. In contrast, iNOS, which is induced by bacterial lipopolysaccharide or cytokines such as interleukin-1 and tumour-necrosis factor-a, produces large amounts of NO which are considered detrimental to the gastric mucosa (Brown *et al.* 1994) and have been found involved in the process of gastric inflammation in man as in rats (Rachmilewitz *et al.* 1994; Nishida *et al.* 1998). However, controversy still exists concerning the deleterious effects of iNOS-produced NO (Wei *et al.* 1995; McCafferty *et al.* 1997; Akiba *et al.* 1998).

Even though the effects of endogenous NO on gastrointestinal inflammation are discussed, there is substantial evidence that intragastric topical application of NO-releasing substances protects the gastric mucosa from damage induced by injurious agents (MacNaughton *et al.* 1989; Kitagawa *et al.* 1990). Nevertheless, this protective effect is dose-sensitive as high doses of exogenous NO can lead to gastric mucosal injury (Lopez-Belmonte *et al.* 1993; Lamarque & Whittle, 1995; Gurbuz *et al.* 1999) similar to what has been previously reported for endogenous NO (Brown *et al.* 1994).

Thus, considering nitrate daily consumption, controversial effects on human health as well as potentiality to induce the release of consequent quantities of NO in the stomach, and the debated role of NO in the modulation of gastric mucosal inflammation, the present study was designed to determine the effects of chronic ingestion of nitrates, in amounts that can be found in our normal daily diet, on an experimentally induced gastritis in rats.

Materials and methods

Animals

Male Wistar rats (Harlan, Gannat, France) weighing between 200 and 250 g at the time of the experiments were housed in polypropylene cages in a temperature-controlled room (21 ± 1°C) under controlled lighting. They were allowed free access to water and were fed during the night (19.00 to 9.00 hours) with a standard diet (A04; UAR, Epinay-sur-Orge). All protocols were approved by the local Institutional Animal Care and Use Committee.

Effect of potassium nitrate treatment on iodoacetamide-induced gastritis: role of nitric oxide

Fourteen groups of eight rats were treated for 7 d (Table 1). Solutions were administered intragastrically using a gastric feeding tube, except where otherwise indicated. The effects of KNO_3 administration on iodoacetamide-induced gastritis were investigated in groups 1 to 4. With this end in view, rats received iodoacetamide (25 mg/kg per d) or distilled water associated (groups 2 and 4) or not (groups 1 and 3) with KNO_3 (125 mg/kg twice daily). The dose of iodoacetamide used was selected from previous dose–response studies (M Larauche, P Anton, L Buéno and J Fioramonti, unpublished results). Administration of iodoacetamide was performed for the duration of 7 d according to Karmeli *et al.* (1996) who showed a maximum increase in gastric myeloperoxidase (MPO) activity and lesions for this period of administration. Groups 5 to 9 were used to assess the involvement of NO in the protective effect of KNO_3 against iodoacetamide-induced gastritis. First, groups 5 and 6, respectively received an NO donor, sodium nitroprusside (SNP; 0·7 mg/kg three times daily) associated or not with iodoacetamide. The dose of SNP used was selected from previous dose–response studies showing the near-maximum inhibition of iodoacetamide-induced gastric mucosal damage (M Larauche, P Anton, L Buéno and J Fioramonti, unpublished results). The three other groups received the NO scavenger, haemoglobin (75 mg/kg twice daily), with either distilled water (group 7), iodoacetamide (group 8), or KNO_3 and iodoacetamide (group 9). Finally, the last four groups (10 to 14) were used to investigate the interrelationships existing between endogenous gastric NO and exogenous NO brought in by KNO_3. Thus, rats received a non-specific inhibitor of NOS, N(G)-nitro-L-arginine methyl ester (L-NAME; 10 mg/kg per d, intraperitoneally), either 30 min before distilled water (group 10), iodoacetamide (group 11), or iodoacetamide and KNO_3 (group 12), or just 5 min after L-arginine or D-arginine (300 mg/kg per d, subcutaneously; groups 13 and 14 respectively). The doses of haemoglobin and L-NAME used were in agreement with previous *in vivo* studies (Fargeas *et al.* 1996; Rouzade *et al.* 1999). Rats were killed 8 d after the beginning of

Table 1. Treatments performed in the fourteen groups of rats

Group number	Treatment
1	Water
2	Water + KNO_3
3	Iodoacetamide
4	Iodoacetamide + KNO_3
5	Water + sodium nitroprusside
6	Iodoacetamide + sodium nitroprusside
7	Water + haemoglobin
8	Iodoacetamide + haemoglobin
9	Iodoacetamide + haemoglobin + KNO_3
10	L-NAME + water
11	L-NAME + iodoacetamide
12	L-NAME + iodoacetamide + KNO_3
13	L-arginine + L-NAME
14	D-arginine + L-NAME

treatments. The stomachs were isolated, removed, and washed with saline (9 g NaCl/l). Segments (20 mm) of the stomach (antrum) were taken off in order to determine MPO and NOS activities and identify histological lesions. Tissues collected for MPO and NOS assays were stored at −80°C until determination.

Collection of plasma and saliva in rats

Two groups of seven rats weighing 200–250 g at the time of the experiment were used. One group received 0·5 ml distilled water intragastrically and the other received 0·5 ml of KNO_3 at the dose of 125 mg/kg. At 30 min after oral administration, rats were anaesthetized using acepromazine (0·6 mg/kg intraperitoneally) and ketamine (120 mg/kg intraperitoneally). At 5 min after anaesthesia, rats received an injection of pilocarpine hydrochloride (0·5 mg/kg intraperitoneally, 0·2 ml). After 5–10 min, whole mixed saliva samples (250 μl) were drawn, and 500 μl of a TCA solution (25 %, v/v) were added. Samples were vigorously vortexed and centrifuged at 3000 ***g*** for 15 min at 4°C. Pellets were discarded and supernatant fractions were stored at −20°C until determination. After saliva collection, 50–60 min after oral administration, the rats' body wall and peritoneum at the ventral midline were incised in order to perform needle puncture in the abdominal aorta. Thus, 1·8 ml of blood was collected using a plastic syringe filled with 0·2 ml of anticoagulant (3·8 % (w/v) sodium citrate). Immediately after collection, whole blood was centrifuged at 800 ***g*** for 15 min at 4°C, and citrated plasma was drawn. In the same way as for saliva, 500 μl of a TCA solution (25 %, v/v) was added to 250 μl citrated plasma. Samples were then vigorously vortexed and centrifuged at 3000 ***g*** for 15 min at 4°C. Pellets were discarded and supernatant fractions were stored at −80°C until assay.

Morphological studies

Pieces of stomach (20 mm long) were fixed in Carnoy's solution, cleared in xylene and embedded in paraffin blocks. Transverse sections (5 μm) were cut and stained with haematoxylin and eosin, and examined by light microscopy in a blind manner. A histological grading scale (microscopic damage score) was used for the determination of the extent of the inflammatory reaction in the gastric mucosa (Fabia *et al.* 1993). Each of the individual variables estimated was graded from 0 to 3, according to the severity of the alterations. The variables were: ulceration; mucosal atrophy; oedema; inflammatory cell infiltration; vascular dilatation.

Myeloperoxidase activity assay

The activity of the enzyme MPO, a marker of polymorphonuclear neutrophil primary granules, was determined in gastric tissue according to a previously described technique (Bradley *et al.* 1982). Segments of stomach were suspended in a potassium phosphate buffer (44 mM-KH_2PO_4, 6 mM-$K_2HPO_4.3H_2O$; pH 6·0) and homogenized on ice using a Polytron (PCU-2; Kinematica GmbH, Lucerne, Switzerland), and followed by three cycles of freezing and thawing. Suspensions were then centrifuged at 9000 ***g*** for 15 min at 4°C. Supernatant fractions were discarded and pellets were resuspended in hexadecyl trimethylammonium bromide buffer (0·5 % (w/v) in the potassium phosphate buffer), a detergent inducing the release of MPO from polymorphonuclear neutrophil primary granules. These suspensions were sonicated on ice (Büchi, Flawil, Switzerland), and again centrifuged at 9000 ***g*** for 15 min at 4°C. Pellets were discarded and supernatant fractions assayed spectrophotometrically for MPO activity and protein contents. Supernatant fractions obtained were diluted in the potassium phosphate buffer containing 0·167 mg *o*-dianisidine dihydrochloride/ml and 0·0005 % (v/v) H_2O_2. MPO from human neutrophils (Sigma, Saint Quentin Fallavier, France; 0·1 U/100 μl) was used as a standard. Changes in absorbance at 450 nm were recorded with a spectrophotometer (Uvikon 922; Kontron Instruments, Saint Quentin en Yvelines, France) every 10 s over 2 min. One unit of MPO activity was defined as the quantity of MPO degrading 1 μmol H_2O_2/min per ml at 25°C. Protein concentrations (g/ml) were determined with a commercial kit of the modified method of Lowry (Detergent Compatible Assay; Bio Rad, Ivry/Seine, France), and MPO activity was expressed as MPO units/g protein.

Determination of nitric oxide synthase activity

Tissue NOS activities were estimated by measuring the rate of conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline (Bush *et al.* 1992). Tissue samples were homogenized on ice in a buffer (pH 7·4) containing Tris-HCl (50 mM), dithiothreitol (1 mM), phenylmethylsulfonylfluoride (1 mM), EDTA (0·1 mM) and two protease inhibitors: leupeptin (23·4 μM) and pepstatin (14·6 μM). After centrifugation (13 500 ***g***, 30 min, 4°C), 100 μl of supernatant fraction was added to a reaction mixture containing 50 mM-Tris-HCl (pH 7·4), 1·58 μM-L-[^{14}C]arginine, 200 μM-NADPH as a co-substrate, 10 μM-FMN, and 10 μM-FAD as prosthetic groups of NOS, 1 mM-tetrahydrobiopterine, 1 mM-dithiothreitol and 50 mM-valine. Determination of total NOS activity was performed by adding 2 mM-$CaCl_2$. iNOS activity was determined in the presence of a Ca chelator, ethylene-glycol-bis(a-aminoethyl)-N,N,N′,N′-tetra-acetic acid (EGTA; 1 mM). cNOS activity was determined by the difference between both activities. After 30 min incubation at 37°C, the enzymic reaction was stopped by adding cold 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (pH 5·5) containing 1 mM-EGTA and 1 mM-EDTA. L-[^{14}C]citrulline formed in the medium was separated by applying the samples to columns containing pre-equilibrated Dowex AG50W-X8, eluting them with water, and measuring the amount of radioactivity with a liquid scintillation Beta counter (Kontron Instruments, St Quentin en Yvelines, France). Protein concentrations (mg/ml) were determined with a commercial kit of the modified method of Lowry (Detergent Compatible Assay; Bio Rad, Ivry/Seine, France) Enzyme activity was expressed as pmol citrulline formed/mg protein per h.

Nitrate and nitrite assays

The levels of nitrates and nitrites were determined in rats' saliva and plasma according to the method of Griess (Grisham *et al.* 1995). Briefly, a 100 μl portion of a sample (saliva or citrated plasma) was incubated for 30 min at 37°C with 50 mM-HEPES buffer, 5 μM-FAD and 0·1 mM-NADPH in a total volume of 500 μl in the presence (nitrites+nitrates levels) or not (nitrites level) of 0·2 units nitrate reductase/ml from *Aspergillus* species. Following the incubation, 5 μl of lactic dehydrogenase (1500 units/ml) and 50 μl of 100 mM-pyruvic acid were added to each tube to oxidize unreacted NADPH. Samples were then incubated for an additional 10 min at 37°C. Premixed Griess reagent (1 ml) (0·2 % (w/v) naphtylethylenediamine+2 % (w/v) sulfanilamide in 5 % (w/v) phosphoric acid) was then added to each tube and after 10 min incubation at room temperature the absorbance of each sample was determined at 543 nm with a spectrophotometer (Uvikon 922; Kontron Instruments, St Quentin en Yvelines, France). $NaNO_3$ (1 mM) and $NaNO_2$ (1 mM) solutions were used as standards. The detection limit of the assay was 1·92 μM and the quantification limit was 6·41 μM.

Chemicals

Iodoacetamide, KNO_3, SNP, haemoglobin, L-NAME, L-arginine hydrochloride, D-arginine hydrochloride, Dowex AG50W-X8 (H^+ form) 100–200 mesh, valine, NADPH, dithiothreitol, phenylmethylsulfonylfluoride, EDTA, EGTA, leupeptin, pepstatin, FMN, FAD, $CaCl_2$, KH_2PO_4, $K_2HPO_4.3H_2O$, hexadecyl trimethyl ammonium bromide, H_2O_2, *o*-dianisidine hydrochloride, lactic dehydrogenase from bovine muscle, $NaNO_3$, $NaNO_2$, naphtylethylenediamine, sulfanilamide, and HEPES and Tris-HCl buffers were purchased from Sigma, Saint Quentin Fallavier, France. L-[^{14}C]arginine was purchased from ICN Biomedicals, Orsay, France. Nitrate reductase from *Aspergillus* species was purchased from Roche Diagnostic Biochemicals, Meylan, France. Phosphoric acid (85 %, w/v) was purchased from Carlo Erba Reactifs, Val de Reuil, France. Solutions used for oral treatments were prepared in distilled water.

Statistical analysis

MPO activity, NOS activity and histological data were analysed by ANOVA followed by Tukey's *post hoc* test for multiple comparisons using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). Saliva and plasma nitrates and nitrites levels were analysed by using Student's *t* test for unpaired values using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). Results are expressed as means and standard errors of the mean, and differences were considered significant at $P<0·05$.

Results

Iodoacetamide-induced gastritis in rats

Iodoacetamide administration for 7 d induced gastric mucosal damage characterized by a significant increase of tissue MPO activity (3112 (SEM 180) *v.* 1206 (SEM 134) MPO units/g protein in controls; $P<0·05$). Histologically, oedema was found in the submucosa, with vascular dilatation and a mild inflammatory cell infiltrate leading to a significant increase in microscopic damage scores (4·56 (SEM 0·50) *v.* 1·30 (SEM 0·30) units for the control group; $P<0·05$) (Fig. 1). Iodoacetamide significantly increased cNOS activity (125 (SEM 17) *v.* 55 (SEM 6) pmol/mg protein per h; $P<0·05$) but did not modify iNOS activity (Fig. 2).

Effect of potassium nitrate treatment on iodoacetamide-induced gastritis

Treatment with KNO_3 suppressed the iodoacetamide-induced increase in gastric MPO activity and in microscopic damage score (1542 (SEM 123) MPO units/g protein and 1·83 (SEM 0·54) units, respectively) (Fig. 1). The increase

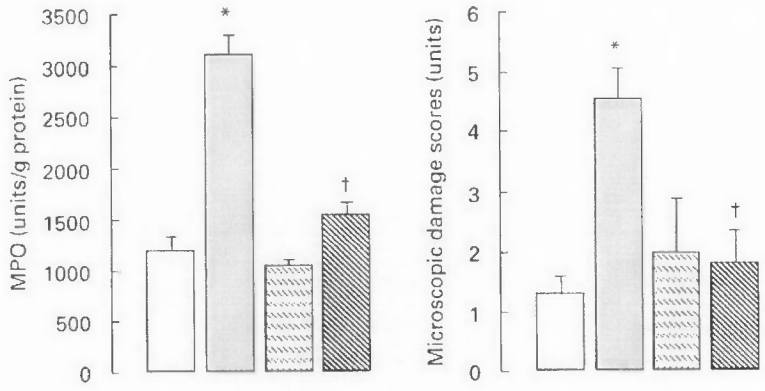

Fig. 1. Effect of KNO_3 on gastric microscopic damage scores and myeloperoxidase (MPO) activity in iodoacetamide-induced gastritis in rats. Mean values for eight rats per group are shown, with standard errors of the mean being represented by vertical bars. * Mean values were significantly different from that of the control group ($P<0·05$). † Mean values were significantly different from that of the group treated with iodoacetamide alone ($P<0·05$). (□), Control group; (▩), iodoacetamide-treated group; (▨), KNO_3-treated group; (▩), KNO_3 + iodoacetamide-treated group.

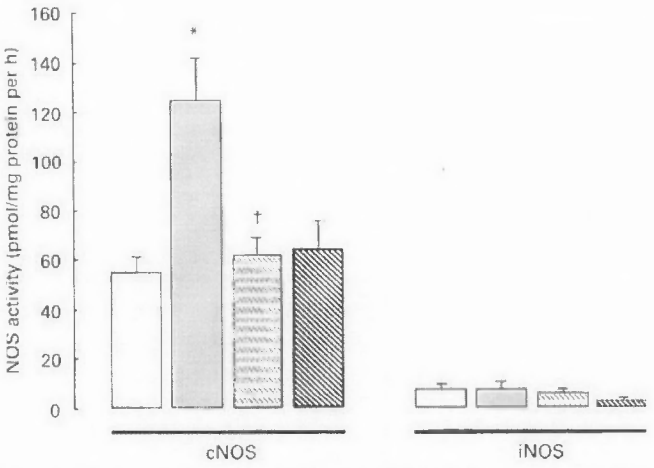

Fig. 2. Effect of KNO_3 on gastric constitutive NO synthase (cNOS) and inducible NO synthase (iNOS) activities in iodoacetamide-induced gastritis in rats. Mean values for five to eleven rats per group are shown, with standard errors of the mean being represented by vertical bars. * Mean value was significantly different from that of the control group ($P<0.05$). † Mean value was significantly different from that of the group treated with iodoacetamide alone ($P<0.05$). (□), Control group; (▩), iodoacetamide-treated group; (▨), KNO_3-treated group; (▩), KNO_3 + iodoacetamide-treated group.

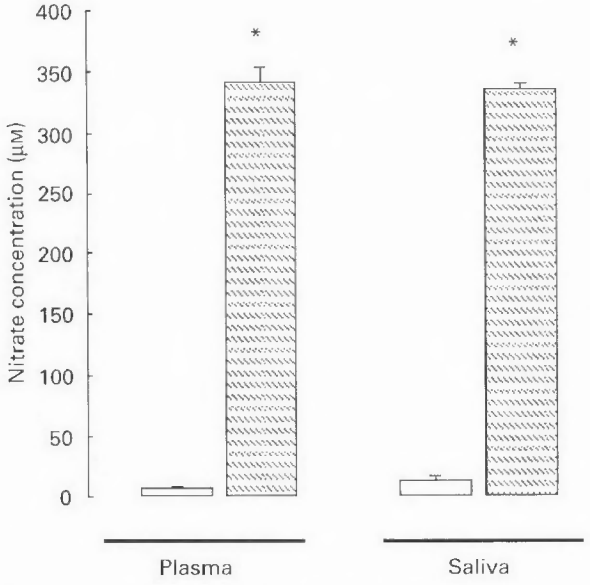

Fig. 3. Effect of KNO_3 on nitrate in the plasma and saliva of rats. Mean values for seven rats per group are shown, with standard errors of the mean being represented by vertical bars. * Mean values were significantly different from that of the control group ($P<0.05$). (□), Control group; (▨), KNO_3-treated group.

in cNOS activity induced by iodoacetamide was abolished by KNO_3 treatment (Fig. 2).

Effect of potassium nitrate treatment on nitrates and nitrites levels in plasma and saliva

In control rats, nitrate levels in plasma and saliva were 7 (SEM 2) and 13 (SEM 4) μM, respectively. Intragastric administration of KNO_3 significantly increased nitrate levels in plasma and saliva compared with control rats (342 (SEM 13) and 336 (SEM 4) μM, respectively) (Fig. 3). Nitrites were undetectable in all samples except in the saliva of all rats treated with KNO_3 where trace amounts below the detection limit of the method were observed.

Involvement of nitric oxide in the protective effect of potassium nitrate against iodoacetamide-induced gastritis

Treatment with the NO donor, SNP, suppressed the increase in gastric MPO activity and microscopic damage score induced by iodoacetamide (1818 (SEM 200) MPO units/g protein and 2·57 (SEM 0·81) units, respectively) (Fig. 4). Treatment with KNO_3 suppressed the iodoacetamide-induced increase in gastric MPO activity and microscopic damage scores, but the co-administration of haemoglobin with KNO_3 restored the increase in gastric MPO activity and damage scores induced by iodoacetamide (Fig. 5). Haemoglobin alone did not modify iodoacetamide-induced gastritis.

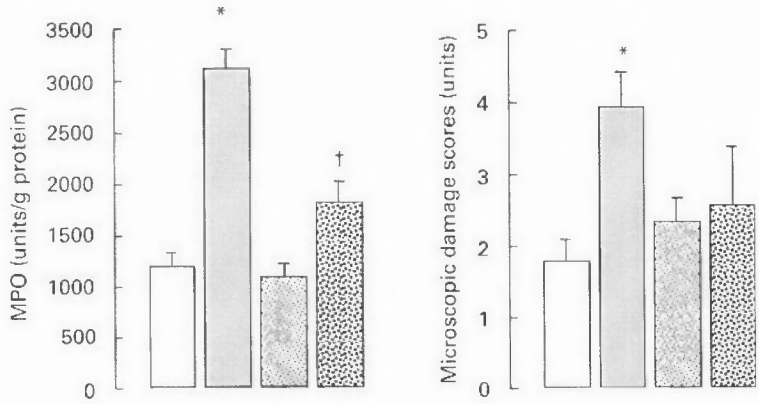

Fig. 4. Effect of sodium nitroprusside on gastric microscopic damage scores and myeloperoxidase (MPO) activity in iodoacetamide-induced gastritis in rats. Mean values for eight rats per group are shown, with standard errors of the mean being represented by vertical bars. * Mean values were significantly different from that of the control group ($P<0.05$). † Mean value was significantly different from that of the group treated with iodoacetamide alone ($P<0.05$). (□), Control group; (▩), iodoacetamide-treated group; (▨), sodium nitroprusside-treated group; (▩), sodium nitroprusside+iodoacetamide-treated group.

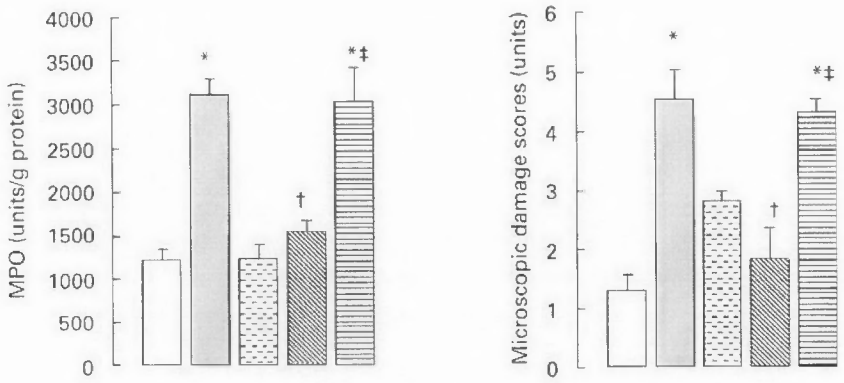

Fig. 5. Influence of haemoglobin on gastric microscopic damage scores and myeloperoxidase (MPO) activity in iodoacetamide-induced gastritis in rats treated with KNO_3. Mean values for eight rats per group are shown, with standard errors of the mean being represented by vertical bars. * Mean values were significantly different from that of the control group ($P<0.05$). † Mean values were significantly different from that of the group treated with iodoacetamide alone ($P<0.05$). ‡ Mean values were significantly different from that of the group treated with KNO_3+iodoacetamide ($P<0.05$). (□), Control group; (▩), iodoacetamide-treated group; (▨), haemoglobin-treated group; (▩), KNO_3+iodoacetamide-treated group; (▤), KNO_3 + iodoacetamide + haemoglobin-treated group.

Interaction between exogenous and endogenous nitric oxide in iodoacetamide-induced gastritis

Repeated administrations of L-NAME once daily for 7 d caused a significant increase in gastric MPO activity (2766 (SEM 258) *v.* 1206 (SEM 134) MPO units/g protein in controls; $P<0.05$). This treatment tended to increase the microscopic damage scores (3·29 (SEM 0·18) *v.* 1·30 (SEM 0·30) units for the control group) but the significance level of $P<0.05$ was not attained (Fig. 6). This effect of L-NAME on gastric MPO activity was antagonized by co-administration of L-arginine (1107 (SEM 106) *v.* 2766 (SEM 258) MPO units/g protein for the L-NAME-treated group; $P<0.05$) but not by D-arginine (2356 (SEM 573) *v.* 2766 (SEM 258) MPO units/g protein for the L-NAME-treated group).

The co-administration of L-NAME with iodoacetamide induced greater gastric MPO activity than L-NAME given alone (4066 (SEM 543) *v.* 2766 (SEM 258) MPO units/g protein for the L-NAME-treated group) and tended to increase the microscopic damage score (5·5 (SEM 0·5) *v.* 3·29 (SEM 0·18) units for the L-NAME-treated group). Treatment with KNO_3 did not prevent the L-NAME-induced aggravation of the gastritis induced by iodoacetamide (Fig. 6).

Discussion

It has been suggested for many years that dietary nitrates were potentially harmful for human health because of their ability to form nitrites and carcinogenic compounds in the stomach (Bruning-Fann & Kaneene, 1993). It has only recently been found that nitrates may play a key role in the mechanism of defence against gastrointestinal and oral pathogens in man and animals (Green, 1995; Duncan *et al.* 1997). Our results extend these later observations by showing for the first time that daily ingestion of an amount of nitrates that can be found in the normal diet protects against an experimental gastritis by releasing NO in the gastric lumen.

In our experiments, gastric mucosal damage was induced by iodoacetamide, a sulfhydryl blocker that has already

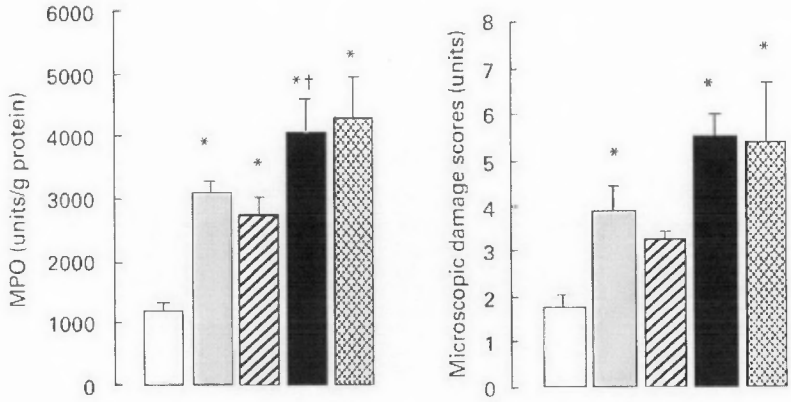

Fig. 6. Effect of N(G)-nitro-L-arginine methyl ester (L-NAME) on gastric microscopic damage scores and myeloperoxidase (MPO) activity in iodoacetamide-induced gastritis in rats treated or not with KNO_3. Mean values for eight rats per group are shown, with standard errors of the mean being represented by vertical bars. * Mean values were significantly different from that of the control group ($P<0.05$). † Mean value was significantly different from that of the group treated with L-NAME alone ($P<0.05$). (□), Control group; (▩), iodoacetamide-treated group; (▨), L-NAME-treated group; (■), L-NAME+iodoacetamide-treated group; (▩), L-NAME+iodoacetamide+KNO_3-treated group.

been shown to induce diffuse gastritis in rats (Lalich, 1962; Yasin & Leese, 1970; Karmeli *et al.* 1996). This agent decreases the concentration of reduced non-protein sulfhydryl compounds in the gastric mucosa (Szabo *et al.* 1981), and consequently alters the structure of the mucus, which is the first barrier of protection of the gastric mucosa. Thus, in our study, daily administration of iodoacetamide for 7 d to Wistar rats induced gastric inflammation characterized by a 2·6-fold increase in gastric MPO activity and a 3·5-fold increase in microscopic damage scores. These results agree with those of Karmeli *et al.* (1996) who showed a 3-fold increase in gastric MPO activity with 7 d of iodoacetamide treatment. The severity of iodoacetamide-induced gastritis was reduced by a 7 d treatment with KNO_3 at a dose of 250 mg/kg per d. Daily nitrate consumption in European countries has been estimated to be about 90–120 mg/person (Cornee *et al.* 1992), corresponding to approximately half of the acceptable daily intake for nitrate in man (World Health Organization, 1962). Taking into account that the acceptable daily intakes for human consumers are calculated by applying a safety factor of 100 to the no-observed effect level determined in animal species (World Health Organization, 1987), it was decided to administer half of the no-observed effect level for nitrates which was estimated at 500 mg/kg per d, expressed in KNO_3 (HP Til, CF Kuper and HE Falke, unpublished results).

KNO_3 administration reduced the increase of gastric MPO activity and abolished the tissue damage induced by iodoacetamide. This reduction of gastric inflammation by KNO_3 was most probably mediated by NO since it was reproduced by an NO donor, SNP, and abolished by an NO scavenger, haemoglobin. In our experiments, nitrate was administered intragastrically to rats. In man, ingested nitrates are absorbed from the upper gastrointestinal tract into the plasma and are then concentrated in saliva (Tannenbaum *et al.* 1976) where they are rapidly reduced to nitrites by anaerobic bacteria (Spiegelhalder *et al.* 1976; Walters & Smith, 1981). It has been suggested that this active transport system of nitrates from blood to saliva is lacking in rats (Vittozzi, 1992; Walker, 1994). However, in rats, orally administered nitrate is rapidly absorbed into the bloodstream from the upper small intestine, and the presence of ^{13}N issued from $^{13}NO_x^-$ administered intravenously has been noted in the saliva of rats (Witter *et al.* 1979). Our results are in agreement with these studies. Indeed, a significant increase of nitrate levels in both plasma and saliva of rats treated intragastrically with KNO_3 was observed; moreover, nitrate levels were identical in the plasma and saliva, confirming the absence of nitrate concentration in the saliva of rats. Even if rats possess nitrate reductase on the tongue (Li *et al.* 1997), significant levels of nitrite in saliva could not be detected. This may be explained by the fact that, in rats, significant nitrite production by tongue bacteria occurs only with very high concentrations of nitrate (Li *et al.* 1997) and also by the technique used to collect saliva. Pilocarpine induces a high flow of saliva, which probably does not allow a sufficient incubation time to have a significant nitrate reduction by tongue bacteria. However, salivary nitrate reduction is not the only way to obtain nitrites in rats. Indeed, nitrate-reducing flora have been identified in the rat stomach (Bockler *et al.* 1983), and the gastric mucosa has been found able to reduce nitrate into nitrite even in germ-free rats (Ward *et al.* 1986). Finally, the possibility of mediation of the effects of nitrate on gastric mucosa by NO is supported by the fact that nitrite is readily transformed into NO in the acidic conditions of the stomach (Benjamin *et al.* 1994).

Some studies have already suggested a protective effect of exogenous NO against gastric mucosal damage (Lopez-Belmonte *et al.* 1993; Calatayud *et al.* 1999; Mourad *et al.* 2000; Potter & Hanson, 2000). However, these effects have been shown with NO donors such as glyceryltrinitrate or isoamylnitrite, systemically administered or in *in vitro* conditions but not after oral ingestion. Our results indicate for the first time a protective action of NO coming from a common dietary component that can be ingested through our daily diet.

Several mechanisms have been proposed to explain the protective role of exogenous NO on gastric mucosa. The most classical effects attributed to NO on gastric mucosa are the stimulation of blood flow and mucus secretion, and the reduction of acid secretion (Pique *et al.* 1989; Brown *et al.* 1993; Kato *et al.* 1998). These three actions can, by themselves, explain the anti-inflammatory effects of NO at the gastric level. However, other hypotheses can be proposed. Because mast cell activation is known to play a key role in gastric inflammation (Nakajima *et al.* 1996, 1997) and exogenous NO has been shown to modulate mast cell degranulation (Gaboury *et al.* 1996; Iikura *et al.* 1998), it is possible that dietary NO exerts its effects by stabilizing mast cells. However, this hypothesis requires further investigation, as mast cell degranulation has not been shown yet to be involved in iodoacetamide-induced gastritis. Another possibility could be that exogenous NO inhibited platelet and polymorphonuclear adherence (Radomski *et al.* 1987; Clancy *et al.* 1992), thus preventing their activation and the consecutive production of oxidants (Clancy & Abramson, 1995; Kubes & Wallace, 1995) whose scavenging function has been shown beneficial in iodoacetamide-induced gastritis (Karmeli *et al.* 1996). Moreover, recent studies have shown the protective role played by haeme oxygenase-1, an antioxidant enzyme, in gastrointestinal inflammation (Cavicchi *et al.* 2000; Wang *et al.* 2000). Since NO donors have been shown to increase haeme oxygenase-1 expression (Hara *et al.* 1999; Cavicchi *et al.* 2000), it could be suggested that dietary NO induced antioxidant and anti-inflammatory effects by targeting haeme oxygenase-1 (Polte *et al.* 2000).

So, considering these possible enzymic modulations of gastric inflammation, an attempt was made to determine the effect of KNO_3 treatment on gastric NOS activity. It is usually postulated that during gastrointestinal inflammation, cNOS activity which is responsible for the maintenance of mucosal integrity is diminished while iNOS activity is overexpressed and produces large quantities of NO leading to mucosal injury. It seems however that the modulation of NOS activity during inflammation differs greatly according to the experimental model used and the type of inflammation (Kimura *et al.* 1997; Nishida *et al.* 1998; Anton *et al.* 2000). A significant increase in gastric cNOS activity was observed, but no

modification of iNOS activity after 7 d of iodoacetamide treatment. cNOS has been found to account for gastric overproduction of NO in uraemic rats (Mendez *et al.* 1997), and excessive levels of NO have been shown to reduce gastric cell viability, alter intracellular glutathione homeostasis and increase generation of intracellular oxidants leading to increased gastric cellular injury (Wakulich & Tepperman, 1997). Moreover, the overexpression of cNOS in portal hypertensive rats is probably responsible for the increased susceptibility of the gastric mucosa to damage (Ohta *et al.* 1997). Then, chronic administration of iodoacetamide could exacerbate the activation of gastric cNOS leading to an overproduction of NO, and then to deleterious effects on the gastric mucosa. Interestingly, KNO_3 treatment abolished the increase in gastric cNOS activity induced by iodoacetamide, suggesting a modulator role of exogenous NO on NOS. A negative feedback of NO on NOS activity has already been shown on cNOS and iNOS enzymes (Griscavage *et al.* 1993; Rengasamy & Johns, 1993), suggesting that the effect afforded by KNO_3 may also be relevant in gastric inflammation where iNOS activity is increased leading to subsequent NO overproduction such as in *Helicobacter pylori*-induced gastritis in human subjects (Franco *et al.* 1999; Fu *et al.* 1999).

So efforts were made to identify interrelations between NOS and dietary NO by using L-NAME, an NOS isoform non-selective inhibitor. L-NAME treatment for 7 d increased gastric MPO activity. This pro-inflammatory effect of L-NAME, already described in the small intestine (Miller *et al.* 1994), unveils a tonic action of NO in the maintenance of gastric mucosa integrity. L-NAME associated with iodoacetamide induced a greater inflammation than L-NAME given alone. This is in agreement with other studies showing that L-NAME aggravates gastric lesions induced by ethanol (Nahavandi *et al.* 1999), stress (Qiu *et al.* 1996) or pylorus ligation (Dixit *et al.* 1999). Exogenous NO brought in by KNO_3 was unable to reduce inflammation induced by iodoacetamide associated with L-NAME. This indicates that exogenous NO can protect the gastric mucosa only in the presence of endogenous NO production.

In conclusion, dietary nitrates exert a protective action on the rat gastric mucosa by producing NO, which can be considered as a complement of endogenous NO. These results confirm the potential therapeutic role of dietary nitrate in human health and highlight the necessity to reconsider its role in our diet.

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Effects of dietary nitrate on oxygen cost during exercise

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Abstract

Aim: Nitric oxide (NO), synthesized from L-arginine by NO synthases, plays a role in adaptation to physical exercise by modulating blood flow, muscular contraction and glucose uptake and in the control of cellular respiration. Recent studies show that NO can be formed *in vivo* also from the reduction of inorganic nitrate (NO_3^-) and nitrite (NO_2^-). The diet constitutes a major source of nitrate, and vegetables are particularly rich in this anion. The aim of this study was to investigate if dietary nitrate had any effect on metabolic and circulatory parameters during exercise.

Method: In a randomized double-blind placebo-controlled crossover study, we tested the effect of dietary nitrate on physiological and metabolic parameters during exercise. Nine healthy young well-trained men performed submaximal and maximal work tests on a cycle ergometer after two separate 3-day periods of dietary supplementation with sodium nitrate (0.1 mmol kg^{-1} - day−1) or an equal amount of sodium chloride (placebo).

Results: The oxygen cost at submaximal exercise was reduced after nitrate supplementation compared with placebo. On an average V_{O_2} decreased from 2.98 ± 0.57 during CON to 2.82 ± 0.58 L min^{-1} during NIT ($P < 0.02$) over the four lowest submaximal work rates. Gross efficiency increased from 19.7 ± 1.6 during CON to 21.1 ± 1.3% during NIT ($P < 0.01$) over the four lowest work rates. There was no difference in heart rate, lactate [Hla], ventilation (VE), VE/V_{O_2} or respiratory exchange ratio between nitrate and placebo during any of the submaximal work rates.

Conclusion: We conclude that dietary nitrate supplementation, in an amount achievable through a diet rich in vegetables, results in a lower oxygen demand during submaximal work. This highly surprising effect occurred without an accompanying increase in lactate concentration, indicating that the energy production had become more efficient. The mechanism of action needs to be clarified but a likely first step is the *in vivo* reduction of dietary nitrate into bioactive nitrogen oxides including nitrite and NO.

Keywords calorie, electron transport, energy expenditure, glycolysis, metabolism, mitochondria, nitric oxide, nitrite, uncoupling protein, V_{O_2}.

Physiological adaptation to exercise involves major cardiovascular and metabolic changes. Oxygen consumption increases dramatically in the active muscles with a parallel increase in the muscle blood flow. In these processes, the endogenous gas nitric oxide (NO) plays an important regulatory role. NO increases blood flow to the muscles and modulates muscular contraction and glucose uptake (for review see Stamler & Meissner 2001). In addition, it is involved in the control of cellular respiration through interaction with enzymes of the mitochondrial respiratory chain (for review, see Moncada & Erusalimsky 2002).

In vitro studies published in the 1990s showed that NO was a modulator of mitochondrial respiration via

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reversible inhibition of cytochrome *c* oxidase (Carr & Ferguson 1990, Bolanos *et al.* 1994, Brown & Cooper 1994, Cleeter *et al.* 1994, Schweizer & Richter 1994). NO may also interact at other sites of the mitochondrial respiratory chain and in the Krebs cycle (for review, see Moncada & Erusalimsky 2002). While this important action of NO has been very well characterized in cell cultures, less is known about its physiological relevance *in vivo* and the effects of NO on cellular respiration during physical exercise. Shen *et al.* (1999) showed that the administration of NOS-inhibitors *in vivo* during submaximal exercise leads to increased oxygen consumption in dogs and Lacerda *et al.* (2006) showed similar results in rats. A majority of studies have been done using NOS-inhibitors, while the effects of administering exogenous NO on exercise are largely unknown. In addition, studies in healthy humans are rare.

The classical means by which NO production occurs is the L-arginine pathway, where NO is synthesized by specific enzymes, the NO-synthases. A fundamentally different alternative way of generating NO was described more recently (Benjamin *et al.* 1994, Lundberg *et al.* 1994, Zweier *et al.* 1995, Weitzberg & Lundberg 1998). In this NOS-independent pathway the inorganic anions nitrate (NO_3^-) and nitrite (NO_2^-) are reduced *in vivo* to form NO. Dietary nitrate (found mainly in green leafy vegetables) (McKnight 1997, Weitzberg & Lundberg 1998) is absorbed from the circulation by the salivary glands, secreted in saliva and partly converted to nitrite in the oral cavity by nitrate-reducing bacteria. Swallowed nitrite can then enter the systemic circulation. Indeed, a recent study showed that ingestion of nitrate resulted in a sustained increase in circulating nitrite levels (Lundberg & Govoni 2004). Further reduction of nitrite into bioactive NO can occur spontaneously in acidic or reducing environments (Benjamin *et al.* 1994, Lundberg *et al.* 1994) but is also greatly enhanced by various proteins and enzymes including deoxyhaemoglobin in blood (Cosby *et al.* 2003), deoxymyoglobin (Shiva *et al.* 2007), xanthine oxidase (Millar *et al.* 1998) and possibly by enzymes of the mitochondrial respiratory chain (for review, see Lundberg *et al.* 2004, Gladwin *et al.* 2005, Lundberg & Weitzberg 2005). NOS-independent NO production seems to complement the endogenous NO production especially during ischaemia and acidosis when oxygen availability is low and the NO synthases operate poorly (Zweier *et al.* 1995, Weitzberg & Lundberg 1998, Duranski *et al.* 2005, Lundberg & Weitzberg 2005). Tissue acidosis and relative hypoxia may be present also during physical exercise and in this metabolic state, bioactivation of nitrite is likely enhanced.

Here, we tested if the administration of dietary nitrate would lead to increased systemic storage pools of nitrite and if this dietary strategy would have an impact on various physiological and biochemical parameters during exercise.

Methods

Subjects

Nine healthy, well-trained (Vo_{2peak} 55 ± 3.7 mL kg^{-1} min^{-1}), males (28 ± 6 years) volunteered for the study. All subjects were trained cyclists or triathletes and well accustomed to the testing procedure. We chose to use well-trained subjects to avoid training effects from the tests such as enhanced Vo_{2peak} or better mechanical efficiency during the submaximal exercise. The protocol was approved by the regional ethics committee in Stockholm and all subjects gave their written consent prior to participation.

Dietary supplementation with nitrate

Our aim with the present study was to investigate the effects of two distinct dietary patterns, one with higher, and one with lower than normal nitrate intake. The study had a double-blind placebo-controlled cross-over design. During two 3-day periods, separated by a washout interval of 10 days, the subjects were instructed to avoid all foods with moderate or high nitrate content (all vegetables, all cured meats, strawberries, grapes and tea). In addition, they were told to restrain from alcohol and tobacco products; otherwise, they were free to eat any food they liked during the 3 days of restricted diet. The subjects were randomized to start with either the ingestion of 0.1 mmol sodium nitrate kg^{-1} bodyweight day^{-1} dissolved in water or an equal amount of sodium chloride (placebo). The daily dose was divided and ingested three times daily. The different solutions could not be distinguished by taste or appearance. The daily nitrate dose corresponded to the amount normally found in 150–250 g of a nitrate-rich vegetable such as spinach, lettuce or beetroot (Lundberg *et al.* 2004). The last dose of nitrate or placebo was ingested in the morning on the day of measurement (see the main tests below). The order between the nitrate supplementation period (NIT) and the placebo period (CON) was balanced. During the washout period, the subjects did not adhere to any specific dietary regime.

Experimental protocol

Measurements were carried out on an electrically braked cycle ergometer (Monark 839E, Varberg, Sweden) that was modified with a racing saddle and

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the pedal system the subjects were familiar with from training. The bicycle ergometer was computer-controlled, permitting a constant work rate regardless of the cadence the subject chose to pedal with. The pedalling cadence was individually chosen in the range of 70–90 rpm but each individual held exactly the same preferred cadence during the NIT and the CON trials. This was to minimize differences in work output due to changes in muscle recruitment patterns.

Pulmonary ventilation (V_E), oxygen uptake (V_{O_2}), CO_2 output (V_{CO_2}) and respiratory exchange ratio (RER) were measured at 10 s intervals by a computerized gas analyser (AMIS 2001, Odense, Denmark) connected to a flow meter which the subjects breathed through via a mouthpiece and a plastic tube. Heart rate (HR) was continuously recorded during the tests with a portable heart rate monitor (Polar S610; Polar, Kempele, Finland). Capillary blood samples (20 μL) were collected from the fingertip and were analysed for lactate ([Hla]) using a Biosen C-Line Sport Analyser (EKF Diagnostics, Magdeburg, Germany). Haemoglobin concentration ([Hb]) at rest was determined with the capillary blood taken from the fingertip and analysed with an Hb-measuring device (Hemocue, Ängelholm, Sweden). Haematocrit (Hct) was determined by centrifuging the capillary blood at 15133*g* for 3 min.

Pre-tests

Each subject attended the laboratory twice within a 2-week period before the first main tests. The first pre-test was carried out to familiarize the subject with the bicycle ergometer and the testing procedure. The subjects did a preliminary test at five submaximal levels with every level lasting for 5 min. There was no rest between the different submaximal levels. V_{O_2} was continuously measured with the AMIS 2001. At the end of each submaximal level capillary blood was taken from the fingertip and analysed for [Hla]. At every work rate the subjects rated their perceived exertion on the Borg's RPE-scale (Borg 1970), and both the central and muscular exertion were rated. After 8 min of recovery, the subject was instructed to cycle for as long as possible at a work rate corresponding to his calculated maximal oxygen uptake (Åstrand & Rodahl 1970). During this test the subjects actual $V_{O_{2peak}}$ was measured and if the subject was able to cycle for longer than 7 min extra power of 20–30 W was added every minute until exhaustion. One and three minutes after the maximal test capillary blood was sampled from the fingertip for analysis of [Hla].

Before the second pre-test, the submaximal levels were adjusted so that they corresponded to 45, 60, 70, 80 and 85% of $V_{O_{2peak}}$. The maximal work rate was also adjusted, if necessary, so that the time to exhaustion was kept between 4 and 7 min.

The main tests

The subjects refrained from heavy exercise 3 days prior to the main tests and avoided all exercise the day before the tests. They were also told to eat their last light meal at least 3 h before the start of the tests. When the subjects came to the laboratory they received their last dose of either placebo or nitrate and were allowed to rest in the supine position for 60 min before the test commenced.

All subjects used a standardized warm up procedure of 5 min of cycling at 100 W followed by 5 min of rest. The submaximal and maximal tests were performed in the same way as the second pre-test with five submaximal work rates lasting 5 min each, without rest between the different levels. Identical work rates were used during the two main tests. Venous blood (9 mL) was drawn at rest 45 min after the last nitrate/placebo-dose was ingested and again immediately after the $V_{O_{2peak}}$ test. The blood was placed in an ice bath and centrifuged within 5 min at 1300 rpm and 4°C. The plasma was separated and kept at −80°C until it was analysed for its nitrate and nitrite concentrations by a chemiluminescence assay as described previously (Lundberg & Govoni 2004).

Statistics and calculations

Results are expressed as mean ± standard deviation (mean ± SD). Submaximal work parameters at multiple work rates were analysed by ANOVA with two-way repeated measures. Paired *t*-tests were used to evaluate the difference between the nitrate and the placebo trials when appropriate. The significance level was set as $P < 0.05$.

Gross efficiency (GE) was defined as the work rate divided by the actual energy expenditure (EE). The EE was in turn calculated with the Brouwer equation (Brouwer 1957).

Delta efficiency (DE) was defined as the increase in work rate, divided by the increase in EE (Gaesser & Brooks 1975). The DE was based on the four lowest work rates which were analysed with linear regression. The oxygen pulse is defined as V_{O_2}/HR.

Results

Blood pressure at rest

The average resting systolic blood pressure was lower after nitrate supplementation (112 ± 8 mmHg) compared with placebo (120 ± 5.9, $P < 0.01$). The diastolic blood pressure was also lower after nitrate (68 ± 5.5

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mmHg) compared with placebo (74 ± 6.8 mmHg, $P < 0.01$). Parts of these findings have been published as a separate communication (Larsen *et al.* 2006).

Blood values

No change was observed in [Hb] at rest (NIT 152 ± 11, CON 153 ± 11 g L^{-1}, $P = 0.87$) or immediately after the Vo_{2peak}-test (NIT 163 ± 13, CON 161 ± 13 g L^{-1}, $P = 0.27$). Nor were there any change in the haematocrit value at rest (NIT 42 ± 4, CON 43 ± 3%, $P = 0.19$) or after the Vo_{2peak}-test (NIT 46 ± 4, CON 47 ± 4%, $P = 0.6$).

Plasma levels of nitrate at rest were 27 ± 6.9 μM in CON and 182 ± 55 μM in NIT ($P < 0.01$). Nitrate levels immediately after the maximal work test were 29 ± 6.1 μM in CON and 175 ± 61 μM in NIT ($P < 0.01$). Plasma nitrate did not change during exercise either in NIT or in CON ($P = 0.8$). Nitrite levels at rest were 124 ± 28 nM in CON and 226 ± 87 nM in NIT ($P < 0.01$). Immediately after the maximal work test the nitrite levels were 111 ± 29 nM in CON and 137 ± 48 nM in NIT ($P = 0.17$). The decrease in nitrite concentrations during exercise was more pronounced in NIT than in CON (Fig. 1).

Submaximal work parameters

After nitrate administration Vo_2 was significantly lower during the four work rates corresponding to 45–80% Vo_{2peak} compared with the placebo period (Fig. 2). On average Vo_2 was 0.16 L min^{-1} ($P < 0.02$, ANOVA) lower in the NIT trials over the 4 submaximal work rates. There was no difference in HR between the NIT and CON trials (see Fig. 2). The oxygen pulse tended to decrease from 21.0 ± 2.0 during CON to 20.3 ± 1.9 mL $beat^{-1}$ ($P = 0.08$, ANOVA). No differences were found between NIT and CON in [Hla] (Fig. 3), V_E, V_E/Vo_2 or RER during any of the submaximal work rates. The average GE increased from 19.7 ± 1.6 during CON to 21.1 ± 1.3% during NIT ($P < 0.01$, ANOVA) over the four lowest work rates. DE increased significantly from 22.1 ± 1.6 during CON compared with 22.9 ± 1.9% during NIT ($P = 0.04$, *t*-test).

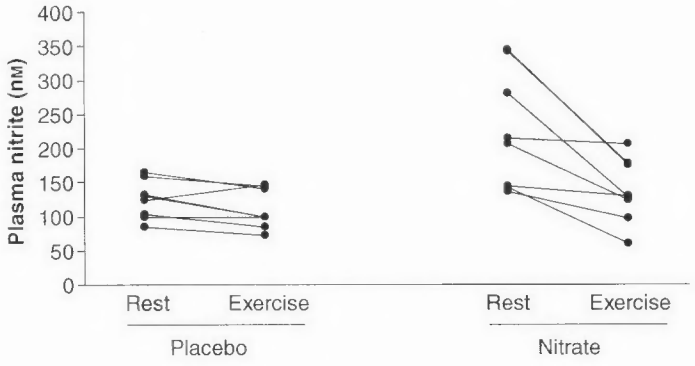

Figure 1 Effects of a dietary supplementation with sodium nitrate or sodium chloride (placebo) on plasma concentrations of nitrite measured at rest and immediately after exercise in seven healthy male volunteers.

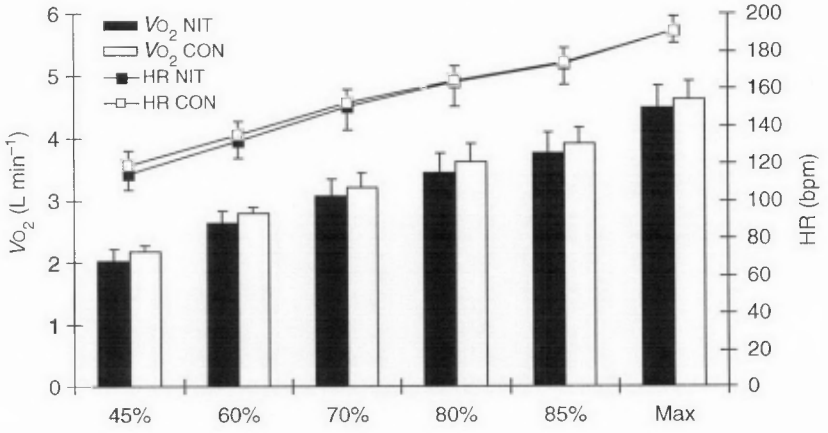

Figure 2 Oxygen consumption (Vo_2) and heart rate (HR) measured at six different work rates after a 3-day dietary supplementation with sodium nitrate (0.1 mmol kg^{-1} day^{-1}, NIT) or an equal amount of sodium chloride (CON). The study had a randomized double-blind crossover design with a washout period of at least 10 days between the tests. There was a significant main effect (*$P < 0.02$, ANOVA) at the four lowest workloads between NIT and CON but no interaction between the different intensities.

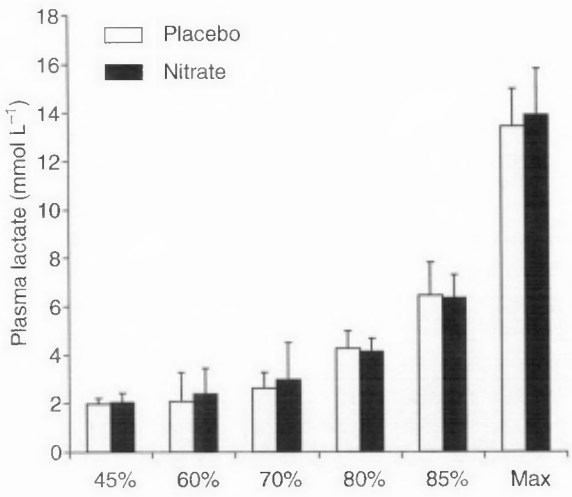

Figure 3 Plasma lactate concentration measured at six different work rates (% of Vo_{2peak}) after dietary supplementation with sodium nitrate (0.1 mmol kg^{-1} day^{-1} for 3 days, filled bars) or an equal amount of sodium chloride (placebo, empty bars).

The chosen cadence varied slightly within the group (mean 82 ± 6 rpm at all submaximal workloads), but was identical for every individual on the two occasions (NIT and CON). Mean work rate was 140 ± 13 W at 45% Vo_{2peak}, 190 ± 17 W at 60% Vo_{2peak}, 229 ± 21 W at 70% Vo_{2peak}, 261 ± 26 W at 80% Vo_{2peak}, and 288 ± 26 W at 85% Vo_{2peak}.

Maximal work capacity

There was no significant difference in the Vo_{2peak} between the NIT and CON trials (4.49 ± 0.44 and 4.61 ± 0.28 L min^{-1}, respectively, $P = 0.29$, *t*-test). These values were also not significantly different from the Vo_{2peak} achieved during the pre-test (4.54 ± 0.32 L min^{-1}). Likewise, no significant differences were noted either in V_{Emax} (NIT 182 ± 21.4 vs. CON 186 ± 21.7 L min^{-1}, $P = 0.5$, *t*-test), HR_{max} (NIT 189.8 ± 7.0 vs. CON 190.3 ± 7.5 beats min^{-1}, $P = 0.94$, *t*-test) or maximal work rate (NIT 360.6 ± 32.8 vs. CON 358.9 ± 32.3 W, $P = 0.35$, *t*-test). There was no difference between NIT and CON in the rating of perceived exertion (Borg RPE-scale) at any workload (submaximal or max).

Cadence at Vo_{2peak} was identical in CON and NIT (86 ± 6 rpm). The workload at Vo_{2peak} was 361 ± 33 W for NIT and 359 ± 32 W for CON.

Comment to the results

In the present study a significantly reduced oxygen demand at the four lowest submaximal work rates was noted after nitrate administration. The fifth work rate, at approx. 85% Vo_{2peak}, was well above the lactate threshold in several subjects and thus the anaerobic energy production became more pronounced. This led to the involvement of accessory muscle groups and a noticeable change in motion pattern. At this work rate the Vo_2 did not reach a stable steady-state level (in the majority of the subjects the Vo_2 increased more than 150 mL min^{-1} during the last 3 min of the work rate) and is therefore unsuitable for the calculation of muscular efficiency. During the four lowest work rates the Vo_2 reached a stable steady-state in all subjects. The reason for including the fifth work rate in the protocol was to receive a lactate value above the lactate threshold and thereby get an indication of changes in the upper part of the lactate curve.

Discussion

One of the fundamentals of exercise physiology is the remarkably tight coupling between oxygen uptake and workload during submaximal cycle ergometer exercise (Åstrand & Rodahl 1970). The consumption of oxygen at a given workload is near identical between different individuals or when measured in the same individual at different occasions. If the findings of the present study hold true, it is possible that this textbook knowledge will have to be revised. We show that dietary supplementation with inorganic nitrate, in an amount achievable through a diet rich in vegetables, results in a reduced Vo_2 during submaximal work and a significant increase in muscular efficiency. These highly surprising effects occurred without any changes in the maximal attainable work rate. We are not aware of any previously described dietary or pharmacological approaches to decrease oxygen consumption during exercise.

At this early stage the mechanism behind the effects of dietary nitrate can only be speculated upon. Nevertheless, there is reason to believe that the observed effects involve initial reduction of nitrate to nitrite. Nitrate itself is believed to be biologically inert and cannot be metabolized by mammalian cells. However, after ingestion nitrate re-enters the mouth via the salivary glands and is effectively reduced by commensal bacteria thereby forming nitrite. In contrast to nitrate, the nitrite ion was recently been shown to possess a wide range of bioactivities (Lundberg *et al.* 2004, Gladwin *et al.* 2005, Lundberg & Weitzberg 2005). In this study, we did indeed note an increase in plasma nitrite after the nitrate treatment period thereby confirming *in vivo* reduction of nitrate as described previously (Lundberg & Govoni 2004, Larsen *et al.* 2006). Another finding in support of nitrite being bioactive was its effective consumption during exercise in contrast to the unchanged levels of plasma nitrate. Ultimately the bioactivity of nitrite is likely related to its

further reduction to NO and possibly other closely related nitrogen intermediates (Gladwin *et al.* 2005, Lundberg & Weitzberg 2005). In addition, it has been recently suggested that nitrite itself may directly affect cellular signalling pathways (Bryan *et al.* 2006). Although probably unlikely, at this stage we also cannot exclude effects of the nitrate anion itself. There are several ways by which biological effects of nitrogen oxides are propagated including alteration of protein function by nitrosylation/nitration or direct binding to protein haeme-moieties as in the prototypic activation of guanylyl cyclase by NO.

If the effects proceed via nitrate reduction to nitrite and then NO formation, how could this then explain our present results? Earlier studies using NOS inhibitors to block endogenous NO production give some indications. NOS-inhibition has been shown to increase submaximal $V\text{O}_2$ in dogs during exercise, independently of the reduction in blood flow (Shen *et al.* 1995, 1999, Ishibashi *et al.* 1998). The increase in $V\text{O}_2$ during NOS-blockade has been explained by the fact that NO affects tissue respiration by reversible inhibition of the respiratory enzyme cytochrome *c* oxidase (Carr & Ferguson 1990, Bolanos *et al.* 1994, Brown & Cooper 1994, Cleeter *et al.* 1994, Schweizer & Richter 1994). Others have related the increased $V\text{O}_2$ during NOS-blockade to an inhibiting effect of NO on proton leakage over the inner mitochondrial membrane (Bohuslavs'kyi *et al.* 2005, Wang *et al.* 2005, Navet *et al.* 2006). If the effects of nitrate were solely due to inhibition of cytochrome *c* oxidase, one would expect an increase in anaerobic metabolism during physical exercise and a larger accumulation of lactate. However, judging from the results this was not the case, as the plasma lactate concentration was near identical after nitrate supplementation compared with placebo. In this context a reduced proton leakage over the inner mitochondrial membrane would fit better with the present results. A smaller waste of protons would indeed improve muscular efficiency at any submaximal work rate.

The studies using NOS inhibitors cited above all imply that endogenous NO is involved in regulation of oxygen consumption but there have been very few attempts to study the effect of exogenous NO delivery. Studies with NO-donors such as nitroprusside and nitroglycerine have shown somewhat diverging results, with decreases in $V\text{O}_2$ in some cases (Loke *et al.* 1999, Recchia *et al.* 1999), no effect in one study (Nunez *et al.* 2005) and increases in other settings (De Backer *et al.* 1996).

Several of the proposed mechanisms for nitrite reduction to NO described above could theoretically come into play during physical exercise. Thus, all these pathways are greatly enhanced during hypoxia and when pH decreases such as in a working muscle (Lundberg *et al.* 2004, Gladwin *et al.* 2005, Lundberg & Weitzberg 2005). Shiva *et al.* (2007) very recently demonstrated deoxymyoglobin-dependent nitrite reduction to NO in rat heart homogenates with a concomitant inhibition of mitochondrial respiration. Another possible pathway includes NO formation by the mitochondria themselves (Kozlov *et al.* 1999) or even simple acidic reduction of nitrite in the working muscle (Zweier *et al.* 1995, Modin *et al.* 2001). Cosby *et al.* (2003) described NO formation and vasodilation from the reaction of circulating nitrite ions with deoxyhaemoglobin in blood. While this latter pathway, and possibly also tissue nitrite reduction, very well might explain the recently described nitrate-induced reduction in resting blood pressure (Larsen *et al.* 2006), it is still not obvious how this NO also would decrease oxygen consumption in the working muscle. Thus, an effective inhibition of mitochondrial respiration (e.g. by deoxymyoglobin-derived NO), would again be expected to result in the accumulation of plasma lactate which was not the case. Even though hypoxia and acidosis can become quite severe in a muscle working at maximal capacity, these metabolic changes are negligible at lower workloads. Yet, we noted a clear lowering of oxygen cost after the nitrate diet already at 45% of $V\text{O}_{2\text{peak}}$. This shows that these newly described effects of nitrate/nitrite are not necessarily augmented by hypoxia/acidosis. In line with this reasoning, it will be of great interest to study if also resting EE is affected by dietary nitrate.

The efficiency of the muscles to produce work has been related to the percentage of type I muscle fibres (Coyle *et al.* 1992) and uncoupling protein-3 content of muscle fibres (Mogensen *et al.* 2006). Other factors that might contribute to the efficiency of movement are anatomical, biochemical and biomechanical features (Williams 1985). Changes in $V\text{O}_2$ at different work rates can originate from differences in substrate utilization. Carbohydrates are slightly more efficient as energy substrate than fatty acids. That is, if more carbohydrates are used as substrate, this will yield a lower oxygen uptake at a given work rate. GE calculations include possible changes in RER and thereby take substrate utilization into account. The improved GE in the NIT-trials indicates better efficiency but even so we cannot exclude that this improved efficiency partly originates from reduced baseline EE. The DE calculations are not dependent on the baseline EE and are also based on all work rates taken together instead of a single work rate at a time as in the GE-calculations. It is therefore plausible to expect DE to be the most valid estimate of muscular efficiency in this case. Indeed, even DE was significantly improved after nitrate supplementation. It is unlikely that the improved efficiency by nitrate comes from mechanical factors. The subjects of this study were all cyclists with many years of experi-

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ence of training and competing. It is improbable that a few visits to the laboratory would change their efficiency during cycling to any noteworthy extent. The fact that the subjects used the same cycling shoes, clip-on pedals and the same seat position as they were used to during training makes this even more unlikely. Most importantly, the randomization procedure used in this study rules out any such differences. Marechal & Gailly (1999) demonstrated a faster relaxing velocity of muscle fibres in *in situ* experiments during administration of an NO-donor, thereby implicating a neuromuscular modulatory effect of NO. It remains to be proven if this can improve the muscular efficiency during cycling.

The finding that the oxygen pulse at a given work rate decreases by nitrate supplementation is a direct effect of the lower oxygen demand at that work rate. However, there is no difference in oxygen pulse at a given absolute oxygen uptake. The lack of effect of nitrate on VE/VO_2 or oxygen pulse indicates that the improved efficiency originates from muscular or mitochondrial adaptations rather than from central adaptations in the heart or the lungs.

In contrast to submaximal work we found no significant effects of nitrate supplementation on the metabolic variables during maximal work. This may be explained by changes in physiological functions at a maximum level of effort compared with a submaximal level. During maximal or near maximal work, the modulatory physiological effects by nitrate are likely to be overridden by emergency reactions. However, we cannot exclude that the lack of effect during maximal work is due to a depletion of the systemic nitrite stores. In addition, we cannot exclude that there is a true difference also at the highest workloads that would have been revealed in a larger group of study subjects.

There are a number of remaining questions that need to be addressed in future studies besides revealing the exact mechanism of action for the effects of dietary nitrate. First, the importance of gender, age, physical fitness and disease is unclear as we only included young healthy well-trained men. Second, we do not know if the effects are limited to physical exercise or if nitrate also modulates resting energy expenditure, and we also need to explore if the effect is specific for a certain tissue or a population of mitochondria. Third, it is possible that an 'NO-like' bioactivity such as that of nitrate, also could effect substrate utilization. Fourth, nitrate intake varies greatly in the population and between different cultures and it will be important to establish the dose-effect ranges and the duration of effects.

In summary, our findings show a lower oxygen cost during submaximal work after dietary supplementation with nitrate, in amounts achievable through a diet rich in vegetables. This remarkable effect occurred without an accompanying increase in plasma lactate, indicating that the energy production had become more efficient. The mechanism of action and main targets need to be clarified but the process likely involves *in vivo* reduction of nitrate into bioactive nitrogen oxides including nitrite and NO.

Conflict of interest

The authors declare no conflict of interest.

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